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The Role of the Ternary Complex Factors in T cell Development and Function

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy from the University of London

2007

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All experiments in this thesis were performed by myself with the exception of the histological staining, and determination of colitis scores presented in Figure 5.6 and the immunoprecipitation analysis presented in Figure 5.3B.

Jane Willoughby

Abstract

Regulatory T cells (T_{regs}) play an important role in immune regulation. Their development in the thymus requires interaction of their TCR with self-peptide-MHC and the induction of Foxp3. The downstream signals from the TCR that lead to commitment to the regulatory lineage and subsequent up-regulation of Foxp3 are unclear. The development of regulatory T cells has been shown to occur at the DP stage of thymocyte development where positive and negative selection occurs and it is thought that T_{regs} differentiate from positively selected thymocytes. The three classical MAPK cascades and their targets have been implicated in both positive and negative selection. Thus they represent an opportunity to gain further insights into the development of regulatory T cells. Here I have compared the requirements of positive selection and regulatory T cell development through the use of knockout and transgenic animals defective in Raf signalling, and components of the SRF regulatory network including the ternary complex factors (TCFs) and SRF itself. Whilst the TCF deficient mice display severe defects in positive selection, T_{reg} development was unimpaired. However depletion of SRF resulted in a complete block in positive selection and T_{reg} development suggesting that positive selection consists of both TCF-dependent and TCF-independent events. Inhibition of Raf signalling by the dominant interfering DN Raf derivative reduced both $Foxp3^+$ and $Foxp3^-$ $CD4^+$ populations. TCR crosslinking efficiently induced ERK activation in regulatory T cells but induction of the TCF target gene was impaired. Nevertheless, both TCF-deficient and DN Raf $CD4^+CD25^+$ T_{regs} effectively suppressed $CD4^+CD25^-$ T cell proliferation *in vitro*. Finally SAP-$1^{-/-}$ $CD4^+CD25^+$ T_{regs} are functional in an *in vivo* model of colitis. Thus the signalling requirements for development of T_{regs} in the thymus are distinct from those required for conventional T cell positive selection.

To my husband

Acknowledgements

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This work would not have been possible without the help of past and present members of the LRI FACs lab, who sorted millions of cells for a small number of experiments. Also I would like to thank the members of the equipment lab including Graham Clark for advice and help with the real-time RT-PCR experiments.

A very large thank you also has to go to members of the animal unit at the LRI, who performed all injections, and the animal technicians at Clare Hall who looked after all breeding lines and did their best to cope with the problems some our mice posed.

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Publications

Some of the work here has been published in the following article:

Willoughby, J.E., Costello, P.S., Nicolas, R.H., Robinson, N.J., Stamp, G., Powrie, F., and Treisman R. (2007) Raf Signaling but not the ERK effector SAP-1 is Required for Regulatory T cell Development. J. Immunology 179:6836-6844

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Abbreviations

DC	dendritic cells
DN	double negative
DN Raf	dominant negative Raf
DP	double positive
KO	knockout
PDBu	phorbol dibutyrate
p-ERK	phosphorylated ERK
SP	single positive
SRF	serum response factor
TCF	ternary complex factor
TCR	T cell receptor
T_{reg}	Regulatory T cells
WT	wild type

1 Introduction

The development and function of an effective immune system is essential to protect against pathogenic viruses, microorganisms and parasites. The mechanism by which an immune repertoire can be generated which is capable of engaging this myriad of insult but at the same time is "educated" not to attack self and result in autoimmunity is a central question in immunology. The thymus is the site where this 'education' takes place. However, there are additional mechanisms that operate to protect against immune responses to self. One such mechanism is the action of regulatory T cells. Experiments presented in this thesis are designed to add to our understanding of the development and function of this important class of T cell. I focus on the role of signalling pathways, particularly the SRF network in this process. The ternary complex factors (TCFs), part of the SRF network, are targets of the MAPK pathway so I will first provide an overview of this signalling cascade before detailing current knowledge of SRF network components, and finally detailing our knowledge of T cell development.

1.1 MAPK cascades

The mitogen-activated protein kinase (MAPK) cascades are evolutionary conserved signalling cascades that have shown to be important for cell proliferation, cell cycle, differentiation and cell death depending on the cell type and stage of development (reviewed in Raman et al., 2007). The cascades are initiated by the activation of MAPK kinase kinases (MKKKs) usually in response to receptor-tyrosine kinase signalling. The MAPKKKs then activate MAPK kinases (MKKs) through phosphorylation that in turn activates the MAPKs by dual phosphorylation of threonine and tyrosine residues in the MAPK activation loop. It has been shown that specific MKKs target specific families of MAPKs. The MKKs MEK1 and MEK2 activate the ERK1/2 kinases, whilst MKK4 and 7 activate JNK (Derijard et al., 1995; Ganiatsas et al., 1998; Tournier et al., 1997) and MKK3, 4 and 6 are capable of activating p38 MAP kinase (Derijard et al., 1995; Raingeaud et al., 1996) (Figure 1.1).

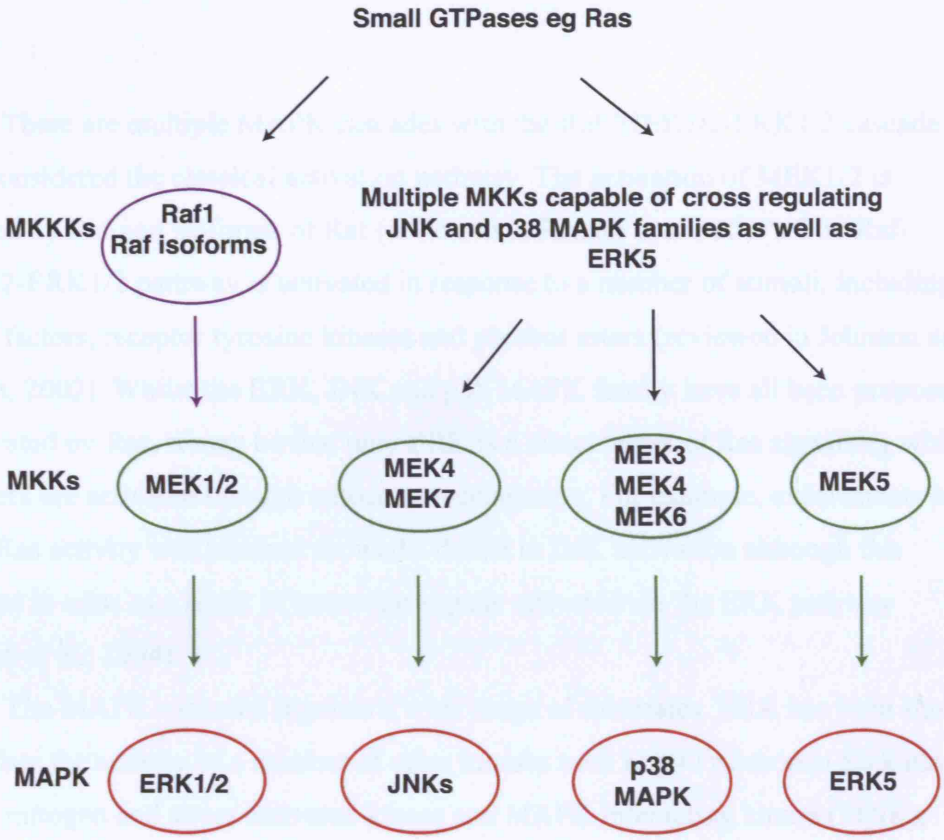


Figure 1.1. MAPK signalling cascades. MAPK cascades are initiated through receptor activation of small GTPases such as Ras. These then activate the MAPK kinase kinases (MKKKs), which in turn activate the MAPK kinase (MKKs), which subsequently activate the MAPKs. The clasical ERK1/2 pathway is shown alongside the JNK, p38 MAPK and ERK5 cascade.

The MKKs themselves are activated by numerous MKK kinases after being activated by small GTPases such as Ras and Rho. Other members of the ERK family have been identified and the MKKs and MKKKs which regulate the activity of these MAPK are being investigated (reviewed in Bogoyevitch and Court, 2004).

1.1.1 Ras-ERK cascade

There are multiple MAPK cascades with the Raf-MEK1/2-ERK1/2 cascade being considered the classical activation pathway. The activation of MEK1/2 is mediated by Raf and isoforms of Raf (reviewed in Raman et al., 2007). The Raf-MEK1/2-ERK1/2 pathway is activated in response to a number of stimuli, including growth factors, receptor tyrosine kinases and phorbol esters (reviewed in Johnson and Lapadat, 2002). Whilst the ERK, JNK and p38 MAPK family have all been proposed to be activated by Ras, it may be that only ERK is a direct target of Ras signalling whilst the others are activated through autocrine mechanisms. For example, experiments in which Ras activity was blocked showed a defect in JNK activation although this appeared to arise as a result of autocrine signals activated via the ERK pathway (Minden et al., 1994).

The MAPK cascades regulate a wide range of substrates. ERK has been shown to regulate the activity of a number of other kinases such as p90 ribosomal S6 kinase (RSK), mitogen and stress activated kinase and MAPK interacting kinase (MNK), whilst also having direct effects on transcription factors such as the Ternary Complex Factor family of transcription factors and AP-1 (reviewed in Raman et al., 2007). With this cascade being important in many cellular processes, the regulation of this cascade must be tightly controlled. Some of the insights into the role and function of this cascade have come from studies using the MEK inhibitor UO126 however, some of these data must be interpreted with the caveat that this molecule is also capable of inhibiting the ERK5 signalling cascade (Kamakura et al., 1999; Mody et al., 2001).

One question is how does this signalling cascade determine whether upon activation of ERK, ERK should then target other kinases, regulate transcription or activate a mixture of transcription factors and kinases in order to achieve a specific cellular function. The Ras-Raf-ERK pathway has been shown to result in different outcomes within the same cell as well as resulting in different outcomes due to cellular context. For instance the Raf-Ras-ERK pathway has been shown to be important for

proliferation but it has also been implicated in apoptosis (Huser et al., 2001; Mikula et al., 2001). Interestingly Raf inhibition of apoptosis does not require its catalytic domain and appears to be mediated through its interaction with the pro-apoptotic kinases MST2 and ASK1 (Chen et al., 2001; O'Neill et al., 2004). Understanding the regulation of this cascade will provide important insights into how different cellular outcomes are achieved through the Ras-Raf-ERK pathway.

There is also the question of the activity of the different Raf isoforms A-Raf, B-Raf and C-Raf or Raf-1. Whilst it has been shown that Ras activates Raf and subsequently ERK, this is a simplistic interpretation (reviewed in Wellbrock et al., 2004). There are four isoforms of Ras which bind Raf with variable efficiencies (Weber et al., 2000). Additionally, whilst B-Raf can be activated via interaction with Ras alone, C-Raf and A-Raf require synergistic activation with Src for maximal activity (Marais et al., 1997). Furthermore the different isoforms appear to have distinct physiological roles: A-Raf$^{-/-}$ animals die between 7 and 21 days after birth due to neurological and gastrointestinal defects (Pritchard et al., 1996); B-Raf$^{-/-}$ animals die *in utero* and the embryos were found to have vascular defects as well as being smaller than WT littermates as determined by embryonal weight (Wojnowski et al., 1997); and C-Raf$^{-/-}$ animals also die *in utero* with their livers containing high numbers of apoptotic cells (Mikula et al., 2001). Whilst most of our understanding of the functions of Raf has come from studies with C-Raf, it has been proposed that B-Raf may be the main regulator of the Ras-Raf-ERK cascade in cells due to the fact that the loss of B-Raf in mouse embryonic fibroblasts resulted in a more severe block of MEK1 activation than the loss of A-Raf or C-Raf (reviewed in Wellbrock et al., 2004). Interestingly a study of A-Raf and C-Raf in neonatal rat heart showed that different stimuli are capable of activating specific isoforms, suggesting that the particular Raf isoform used may be important in directing specific cellular outcomes (Bogoyevitch et al., 1995).

Increasing evidence supports the idea that scaffold proteins and phosphatases are important in regulating signals as they pass through the cascade (reviewed in Kolch, 2005; Liu et al., 2007; Lang et al., 2006). Scaffold proteins can recruit different components of a specific pathway simultaneously. This close proximity of components allows specific complexes of particular pathway components to form, and thereby efficient phosphorylation and pathway activation. The first scaffold protein identified for the MAPK cascade was the Ste5 protein in yeast. This was shown to selectively tether the MKKK, MKK, and MAPK of the yeast mating pathway (reviewed in Elion, 2001). Other potential scaffold proteins have since been implicated in the Raf-ERK

cascade; one of these is the kinase suppressor of Ras (KSR) protein. Whilst this protein has some homology to Raf, including a putative kinase domain, it has been proposed that its main function is to act as a scaffold protein (Kortum and Lewis, 2004). KSR has shown to be constitutively associated with MEK1 but may also associate with ERK1/2 and Raf (reviewed in Morrison, 2001). Another scaffold implicated in regulating Raf is CNK, which has been shown to link Src to Raf activation (Ziogas et al., 2005). There are at least three isoforms of CNK in mammals with potentially distinct functions and which have also been implicated in other pathways such as the regulation of the MST1/2 kinases and apoptosis (Rabizadeh et al., 2004), in addition to being involved in the regulation of the Rho GTPases' transcriptional activities (Jaffe et al., 2004).

Some of the distinct outcomes achieved though the same signalling cascade can also be explained by the kinetics of MAPK activation. The system for which this is best described is the differentiation vs. proliferation choice seen in PC12 cells. Activation by NGF results in the differentiation of PC12 cells, whereas treatment with EGF results in proliferation (reviewed in Marshall, 1995). The activation of PC12 cells by NGF induces a persistent activation of ERK, whereas EGF only induces a transient activation (Traverse et al., 1992). Over-expression of the EGF receptor mimicked NGF induced differentiation (Traverse et al., 1994) which, interpreted with the data that the EGF receptor is more rapidly internalised (Countaway et al., 1992), suggested that it was the active number of cell surface receptors which determines the kinetics of ERK activation. Whilst the knowledge of other regulatory proteins within the Raf-MEK-ERK cascade indicates that regulatory proteins may influence the kinetics of activation, the PC12 model demonstrates that different outcomes can also be determined by duration of signal.

Furthermore, it has also been proposed that compartmentalisation of the signal can be particularly important to the outcome (reviewed in Murphy and Blenis, 2006; Mor and Philips, 2006). Ras signalling has been demonstrated to occur not just at the plasma membrane but also at the golgi and endoplasmic reticulum resulting in differential activation of signalling pathways (Chiu et al., 2002). Visualisation of Ras during thymocyte development showed that the location of Ras correlated with distinct outcomes (Daniels et al., 2006).

The MAPK cascades have been implicated in developmental processes (reviewed in Chang and Karin, 2001). Knockouts of some of the MAPK cascade components results in embryonic lethality. $Mek1^{-/-}$ mice die *in utero* possibly through placental defects, whilst analysis of $Mek1^{-/-}$ fibroblasts revealed a defect in migration

(Giroux et al., 1999). Deletion of ERK2 also resulted in embryonic lethality that occurred around embryonic day 6.5 or embryonic day 11.5 depending on the deletion strategy used (Yao et al., 2003; Hatano et al., 2003). Deletion of exon 2 resulted in a lack of mesoderm formation whilst deletion of exon 3 resulted in placental abnormalities. In contrast the $ERK1^{-/-}$ mice were viable and were initially reported to have a defect in thymocyte development (Pages et al., 1999). However more detailed analysis demonstrated that this was only a minor defect and that ERK2 can act redundantly during this process. Furthermore loss of ERK2 specifically in T cells resulted in a severe block in thymocyte development, which when combined with ERK1 deletion resulted in a near complete block of T cell development (Fischer et al., 2005). Further insight into the role of the MAPK cascades has been possible through the use of dominant negative transgenes, particularly with regards to thymocyte development (as will be discussed in section 1.4).

1.2 Regulation of Signalling to the Serum Response Factor

The serum response factor (SRF) is a widely expressed MADS-Box transcription factor. Deletion of SRF resulted in an embryonic lethal phenotype and identified SRF as being important in mesoderm formation (Arsenian et al., 1998). Targeted disruption of SRF in the heart resulted in lethal cardiac defects (Parlakian et al., 2004), whilst lymphocyte targeted disruption resulted in a severe defect in positive selection (Fleige et al., 2007). The pleiotropic effects of SRF deletion are consistent with its ability to regulate a large number of genes. SRF is capable of interacting with a number of transcriptional partners in order to generate specificity of gene transcription (Philippar et al., 2004; Chen and Schwartz, 1996; Belaguli et al., 2000; Miralles et al., 2003; reviewed in Posern and Treisman, 2006; Pipes et al., 2006).

The two most extensively studied signalling pathways which impact on SRF mediated gene transcription are the Ras-ERK cascade and the Rho signalling pathway. The effects of the Rho signalling pathway are mediated by members of the Myocardin Related Transcription Factor Family (MRTFs) (Miralles et al., 2003; Wang et al., 2001). Whilst myocardin itself does not seem to be regulated by Rho signalling, the other members of this family such as MAL/MRTF-A and MKL2/MRTF-B are regulated by this pathway (Kuwahara et al., 2005; Miralles et al., 2003). The interaction

of the MRTFs with SRF results in transcription of muscle-specific genes and cytoskeletal genes, such as actin and vinculin (Miralles et al., 2003; Gineitis and Treisman, 2001).

The Ras-ERK cascade activates the Ternary Complex Factor (TCF) family of transcription factors (see below) to modulate gene transcription mediated by SRF (Gille et al., 1995; Marais et al., 1993; Shaw et al., 1989). Regulation by the TCFs primarily induces expression of immediate early genes such as c-fos and Egr-1 (Gineitis and Treisman, 2001). Whilst biochemical analysis showed that the MRTFs and TCFs compete for an overlapping binding site on SRF (Murai and Treisman, 2002; Miralles et al., 2003; Wang et al., 2004), differences in the mechanism of binding at this overlapping site have also been described (Zaromytidou et al., 2006).

1.3 Ets domain transcription factors

The Ets family is a large family of DNA binding proteins; 27 Ets domain proteins have been identified in humans which can be further subdivided into more highly related sub-groups (Hollenhorst et al., 2007). Many of the Ets proteins recognise broadly similar DNA binding sites, yet they appear to have specific biological functions and disruption of murine Ets genes has identified unique phenotypes (Hollenhorst et al., 2004; Zhou et al., 2005; Hollenhorst et al., 2007). Examination of Ets promoter target sites revealed both specific and redundant occupancy (Hollenhorst et al., 2007). Part of this specificity may be due to their interaction with specific transcription factor partners, although how these specific interactions are determined is not completely understood (Sharrocks, 2001). Additional specificity may also be generated through tissue specific expression.

1.3.1 The Ternary Complex Factor (TCF) family of transcription factors

The Ternary Complex Factor (TCF) family of transcription factors are part of the Ets transcription factor family. The TCF family consists of Elk-1, SAP-1 and Net.

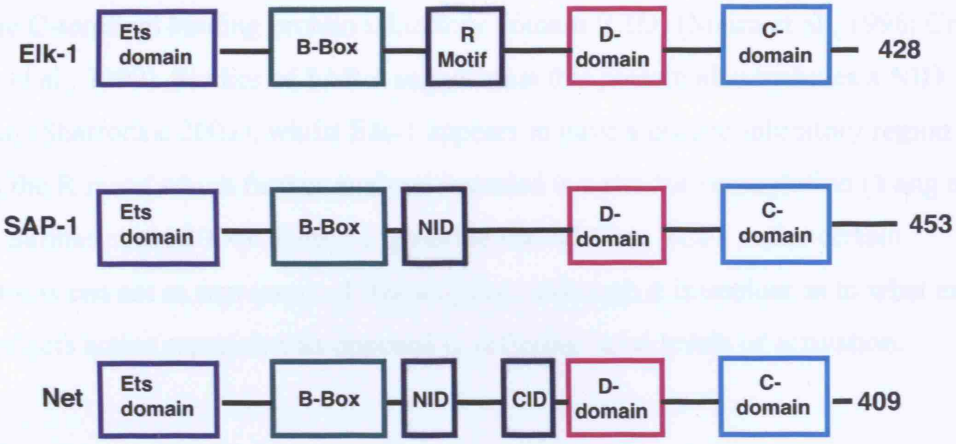


Figure 1.2. Ternary Complex Factors (TCFs) contain four highly conserved domains. The TCFs contain four highly conserved regions: the A domain includes the Ets domain which binds to the Ets consesus site in DNA; the B-Box which interacts with the serum response factor; the D-domain which contains docking sites for the MAPKs and the the C-domain which contains the target sites for MAPK phosphorylation. Net also includes well defined inhibitory domains called Net inhibitory domain (NID) and C-terminal binding protein (CtBP) inhibitory domain (CID). The NID is also thought to be present in SAP-1. Elk-1 also contains the R motif which is a sumoylation site.

All three contain four conserved domains including the A domain which contains the Ets DNA binding domain, the B-box which interacts with the serum response factor, the C- domain which contains sites of MAPK phosphorylation and the D domain which contains the docking sites for the MAPK (Figure 1.2) (reviewed in Treisman, 1994; Buchwalter et al., 2004).

The TCFs have been suggested to be both activators and inhibitors of transcription. Net in particular has been shown to repress transcription, whilst repressive effects have also been demonstrated for Elk-1 (Giovane et al., 1994; Yang et al., 2001). Net has been shown to have two inhibitory domains; the Net inhibitory domain (NID) and the C-terminal binding protein inhibitory domain (CID) (Maira et al., 1996; Criqui-Filipe et al., 1999). Studies of SAP-1 suggest that this protein also includes a NID domain (Sharrocks, 2002); whilst Elk-1 appears to have a unique inhibitory region called the R motif which further analysis revealed is a site for sumoylation (Yang et al., 2002; Salinas et al., 2004). Thus it is possible that all three TCFs under certain conditions can act as repressors of transcription, although it is unclear as to what extent this reflects active repression as opposed to differing basal levels of activation.

1.3.1.1 Activation of the TCFs

Despite the potential inhibitory function of the TCFs described above, upon signalling the predominant role of the TCFs is to act as activators of transcription. Ras-ERK signalling, as well as augmenting the transcriptional activities of SAP-1 and Elk-1, converts Net into a transcriptional co-activator (Criqui-Filipe et al., 1999). The TCFs have been shown to be direct targets of the MAPK cascades (Price et al., 1995; Marais et al., 1993; Ducret et al., 2000; Gille et al., 1995; Janknecht et al., 1993; Price et al., 1996; Strahl et al., 1996). It has also been shown that TCF activation in response to signalling can differ between cell types with regards to the MAPKs involved in their activation. For instance, IL-1 signalling activates both Elk-1 and SAP-1 in CHO and NIH3T3 cells. In both cell types, activation of Elk-1 requires JNK activity whilst activation of SAP-1 requires p38 MAPK activity but only in NIH3T3 cells (Whitmarsh et al., 1997). Co-expression studies have also suggested that the ERK5 MAPK preferentially phosphorylates and activates SAP-1 but not Elk-1 (Kamakura et al., 1999). The phosphorylation of the TCFs SAP-1 and Elk-1 has been shown to augment the transcriptional activity of the TCFs by relieving the autoinhibitory interactions

between the Ets domain and carboxy terminus (Gille et al., 1995; Janknecht et al., 1994; Strahl et al., 1996; Shore et al., 1996; Yang et al., 1999).

1.3.1.2 Inhibition of TCF activity

In addition to regulation by phosphorylation, recent studies have shown that Elk-1 and Net are modified by sumoylation and in both cases this modification leads to the repression or enhancement of repression of TCF target genes (Yang et al., 2003; Wasylyk et al., 2005). Sumoylation of Elk-1 was shown to lead to the repression of Elk-1 targets through the recruitment of histone deacetylases to Elk-1 target gene promoters (Yang and Sharrocks, 2004). In this context, MAPK activation of Elk-1 appeared not only to be due to phosphorylation but also as a result of the loss of sumoylation and thus the inhibitory effects of this modification (Yang et al., 2003).

The TCFs have also shown to be inhibited by interaction with the Id proteins by disrupting the formation of the ternary complex with SRF (Yates et al., 1999). This is possibly a negative feedback process as Id proteins have been shown to be up-regulated in response to serum and mitogenic stimulus as immediate early genes and Id3 has shown to be up-regulated in response to the ERK-MAPK cascade (Bain et al., 2001; reviewed in Norton et al., 1998). Furthermore Id3 is up-regulated in response to Egr-1 which is itself a TCF target gene (Bain et al., 2001). De-phosphorylation may also regulate TCF activity. Although this has not been well studied, the calcium-dependent phosphatase calcineurin has been proposed as a physiological phosphatase of Elk-1 (Sugimoto et al., 1997).

1.3.1.3 Biological functions of the TCFs

As targets of the MAPK cascades, the TCFs have the potential to be involved in many cellular processes. To address the physiological functions of the TCFs, knockout and transgenic animals with deficient TCF activity have been generated. Targeted disruption of Elk-1 revealed generally healthy animals with no obvious defects, raising the possibility that the TCFs may have redundant functions during development, with each of them being capable of compensating for the others (Cesari et al., 2004). Examination of Elk-$1^{-/-}$ mice revealed a reduction in the expression of the TCF target

gene c-fos in certain areas within the brain. This suggested that Elk-1 might have a role in selected activities of the brain. In contrast the generation of Netδ mice which express a mutant Net protein, resulted in mice dying shortly after birth due to chylothorax and displayed vascular defects (Ayadi et al., 2001). Furthermore Net was implicated in wound healing and cell migration, possibly through its repression of the plasminogen activator inhibitor 1 (PAI-1) protein (Buchwalter et al., 2005; Zheng et al., 2003). The deletion of SAP-1 resulted in defects in positive selection of developing thymocytes (Costello et al., 2004). The initial SAP-$1^{-/-}$ mice also displayed a phenotype similar to Castleman's disease; including plasma cell infiltration of the liver and salivary glands, high levels of IL-6 in the serum and the presence of DNA antibodies (RT unpublished observations). This phenotype appeared to correlate with MCMV infection but attempts to reproduce this via direct MCMV infection revealed that there was no correlation (J.W. unpublished observations). Thus whilst it is possible that there is some redundancy in function of the TCFs, there is also evidence as described above that they have discrete functions.

1.4 T cell development

The phenomenon of immune tolerance is a striking feature of the immune system. An intricate balance between the requirement for immunity against foreign antigens and the requirement for tolerance of self is maintained through several mechanisms acting at the level of immune cell development and by direct or indirect suppressive interactions between lymphocyte populations.

Immune responses are tightly regulated in order to prevent harmful reactions. As exemplified by *in vitro* differentiation of Th17 and regulatory T cells (see section 1.4.3), the balance between autoimmunity and tolerance can be a fine one. As described below there are several mechanisms involved in the regulation of immune responses, some are instigated via development processes whilst others are initiated in the periphery.

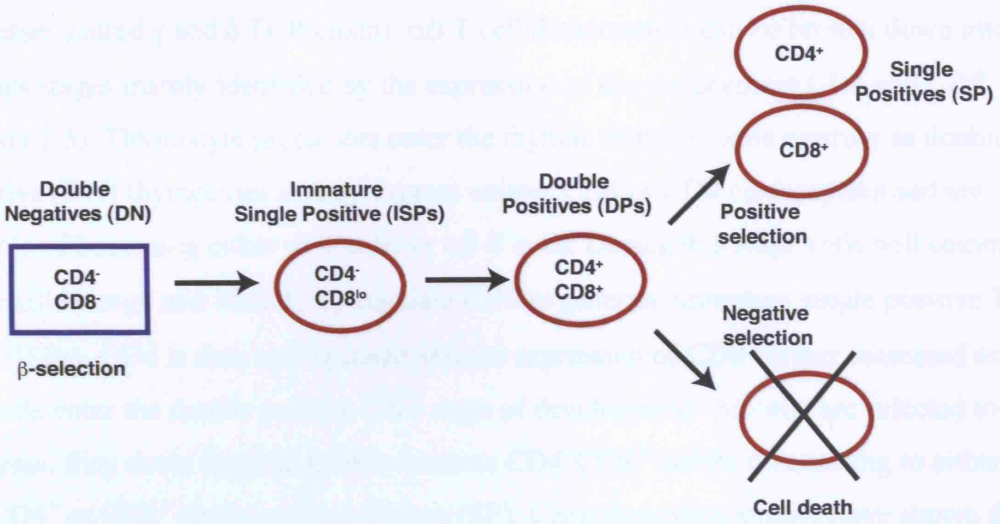


Figure 1.3. αβ T cell development. Thymic precursors enter from the bone marrow as double negative T cells which don't express either CD4 or CD8 co-receptors. At this stage the cells can either become αβ T cells or γδ T cells. αβ T cells undergo β-selection and then partially up-regulate CD8 to become immature single positive T cells (ISPs). CD8 is then further up-regulated along with CD4 to become double positive T cells. These cells have now rearranged the TCR α chain and signalling through the rearranged TCR will determine whether the cells are positively selected to become $CD4^+$ or $CD8^+$ single positive T cells or whether they will die by negative selection.

1.4.1 Overview of αβ T cell development

In order to generate a T cell repertoire that is capable of recognising foreign antigen whilst not containing autoreactive T cells, T cell development is a highly regulated process. In the thymus two distinct populations of T cells develop, identified by the usage of specific T cell receptor (TCR) chains. The predominant population consists of T cells expressing paired α and β TCR chains, whilst a smaller population expresses paired γ and δ TCR chains. αβ T cell development can be broken down into various stages mainly identified by the expression of the co-receptors CD4 and CD8 (Figure 1.3). Thymocyte precursors enter the thymus from the bone marrow as double negative (DN) thymocytes as they express neither CD4 or CD8 co-receptors and are capable of becoming either γδ T cells or αβ T cells. During this stage cells will commit to the αβ lineage and initially up-regulate CD8 to generate immature single positive T cells (ISPs). CD4 is then up-regulated and the expression of CD8 further increased as the cells enter the double positive (DP) stage of development. As cells are selected to progress, they down regulate CD8 to become $CD4^+CD8^{lo}$ before committing to either the $CD4^+$ or $CD8^+$ single positive lineage (SP). Gene disruption studies have shown that this process can be blocked at distinct developmental stages indicating that this is an ordered process. This is reminiscent of checkpoints controlling the cell cycle and thus led to the proposal that thymocyte development consists of several checkpoints at which development can be blocked if the correct signals are not received. Many studies have been performed to elucidate the signalling requirements behind the various developmental stages.

The double negative thymocytes can be further subdivided on the basis of expression of CD44 and CD25 (Figure 1.4). Starting at the DN1 ($CD25^-CD44^+$) stage, cells up-regulate CD25 and progress towards the DN2 stage ($CD44^+CD25^+$). During DN2 rearrangement of the TCRβ chain is initiated and completed in the DN3 ($CD25^+CD44^-$) stage. The TCRβ chain pairs with the surrogate α chain pre-Tα to form the pre-TCR. Cells unable to successfully rearrange the TCRβ chain die via apoptosis whilst those cells which have undergone successful rearrangement undergo several rounds of proliferation and down-regulate CD25 to become DN4 ($CD25^-CD44^-$) T cells (reviewed in Michie and Zuniga-Pflucker, 2002). The successful rearrangement of the TCRβ chain is an integral part of the β-selection checkpoint (which will be discussed

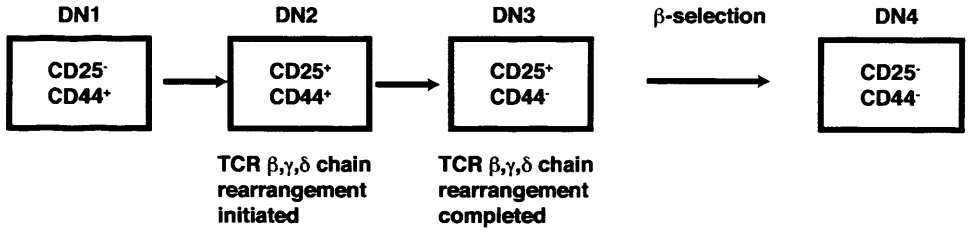


Figure 1.4. Double negative T cell development can be sub-divided by CD44 and CD25 expression. The double negative (DN) developmental stage can be further divided on the basis of CD44 and CD25 expression. The earliest stage, DN1, can be identified as $CD25^{-}CD44^{+}$ cells. CD25 is then up-regulated during the transition to DN2. Rearrangement of the TCR genes *Tcrb*, *Tcrg* and *Tcrd* is initiated in the DN2 stage and completed in the DN3 stage, whereby CD44 has also been down-regulated. Cells with a functional pairing of TCRβ and pre-Tα chains pass through β-selection, undergo several rounds of proliferation and down-regulate CD25 to become DN4 cells

further in section 1.4.1.1). Signals from the pre-TCR then initiate more rounds of proliferation, up-regulation of CD4 and CD8 co-receptors to become double positive thymocytes (DPs), as well as initiating rearrangement of the TCRα chain prior to pairing with the rearranged TCRβ chain to form the mature αβ TCR. A minority of DP thymocytes are then selected to become single positive (SP) thymocytes expressing either CD4 or CD8, a process that is called positive selection.

The successful progression of a DP thymocyte to a SP thymocyte is thought to be determined by the affinity of the interaction between the newly formed αβ TCR and peptide presented by MHC molecules. Some of the rearranged TCRs will be incapable of recognising peptide presented on MHC and will die "by neglect". The remaining T cells express TCRs that are capable of recognising peptide presented by MHC but with varying degrees of affinity. Some of these T cells will express TCRs that have a high affinity for peptide-MHC and, if they were to exit into the periphery, may have the potential to initiate an autoimmune response. The mechanisms for maintaining immune tolerance requires that signals through both the pre-TCR and αβTCR are correctly interpreted as either a survival signal or a death signal. Cells with a high affinity TCR are removed from the T cell repertoire by the process of negative selection (reviewed in Palmer, 2003; Starr et al., 2003), whilst the remaining cells with a relatively weak affinity for interacting with self-peptide-MHC will be positively selected into CD4 or CD8 SP T cells (reviewed in Alberola-Ila and Hernandez-Hoyos, 2003; Basson and Zamoyska, 2000; Germain, 2002; Starr et al., 2003). Recently it has been proposed that it is the process of negative selection which is required for determining the peptide specificity of the mature TCR population (Huseby et al., 2003). It is unclear whether positive and negative selection occurs simultaneously or whether they are sequential. However, it is generally thought that negative selection takes place after positive selection with positive selection occurring in the cortical region of the thymus and negative selection occurring in the medulla region (Bevan, 1977; Speiser et al., 1989, reviewed in Miosge and Zamoyska, 2007; Petrie and Zuniga-Pflucker, 2007; Palmer, 2003; Yin et al., 2006; Ladi et al., 2006). Although it has been suggested that under certain conditions, negative selection may take place prior to positive selection (reviewed in von Boehmer and Kisielow 2006).

1.4.1.1 β-selection

Signals from the TCR result in many varied outcomes depending on the stage of development and previous input. As described above, signals through the TCR during thymocyte development can either lead to death via negative selection or to survival through positive selection, in addition to being required for proliferation. This can also be observed in the periphery where TCR signals can lead to proliferation, differentiation, anergy or cell death. Thus an obvious question is how these different outcomes are derived from activation of the pre-TCR and TCR. Transgenic studies have played an important role in furthering the understanding of how this may occur. One checkpoint during thymocyte development is β–selection which prevents T cells with a non-functioning pre-TCR from developing further (Dudley et al., 1994). This process occurs during the transition from the DN3 to DN4 stage of thymocyte development following the rearrangement of the TCRβ chain and subsequent pairing with the pre-Ta chain to form the pre-TCR. The survival, proliferation and differentiation of the immature thymocytes require pre-TCR signalling. The remaining β chain allele needs to be prevented from rearrangement in a process termed allelic exclusion and this has also been shown to require pre-TCR signalling. Signalling through the pre-TCR is thought to occur in a ligand independent manner, through accumulation of the pre-TCR in lipid rafts (Saint-Ruf et al., 2000; Irving et al., 1998; reviewed in Miosge and Zamoyska 2007). Many of the transcription factors and adaptor proteins which are activated are similar to those seen in TCRαβ signalling in mature thymocytes (Zuniga-Pflucker et al., 1993 and reviewed in Michie and Zuniga-Pflucker, 2002).

Transgenic studies have revealed differing requirements for allelic exclusion and the proliferation and differentiation of immature thymocytes. Loss of function studies have shown a requirement for the proximal protein tyrosine kinases Lck and Fyn, the Zap70 family of kinases and the adaptor proteins LAT and Slp76 (Molina et al., 1992; Groves et al., 1996; van Oers et al., 1996; Cheng and Chan, 1997; Zhang et al., 1999; Pivniouk et al., 1998; and Clements et al., 1998). The role of these proteins was further confirmed in $RAG^{-/-}$ studies in which the block at DN3 due to the inability of the cells to rearrange their TCR chains was rescued by active versions of many of the above proteins. In addition active Ras was also able to rescue this phenotype (Mombaerts et al., 1994; Gartner et al., 1999; Iritani et al., 1999; and Swat et al., 1996). Interestingly

while most of the above-mentioned proteins were also required for allelic exclusion, Ras was only required for proliferation and differentiation of the pre-T cells (Gartner et al., 1999; Iritani et al., 1999). These data highlight how processes that are undertaken at similar times and required to be completed before correct progression can be initiated through the pre-TCR but make use of distinct signalling pathways. It was also shown that Rho has an important role in the differentiation of pre-T cells, even when a functional TCR was expressed; mice in which Rho activity had been blocked by transgenic expression of the Rho-specific inactivator C3 transferase displayed a block at the DN3 stage of development (Cleverley et al., 1999).

The transcription factors which may be involved in the differentiation and proliferation aspects of β-selection have also been investigated. There is evidence that the E-proteins and Id proteins have significant roles in mediating the signals from the pre-TCR. Disruption of E2A resulted in a block prior to β-selection; however this protein is also important in preventing differentiation of DN to DP thymocytes in the absence of β-selection (Bain et al., 1997; Engel and Murre, 2001). It is likely that Id3, an inhibitor of the E-proteins, is responsible for blocking the inhibitory effects of E2A in the presence of pre-TCR signalling as it has been shown to be up-regulated in a pre-TCR-dependent manner (Engel and Murre, 2001). In mature thymocytes, Egr-1 has been shown to up-regulate Id3 and whilst this link has not been shown in pre-T cells, expression of Egr-1 in $RAG^{-/-}$ mice promoted the development of immature single positives (ISP) suggesting that it is a likely candidate to link the RAS-ERK cascade implicated above to Id3 (Bain et al., 2001; Miyazaki, 1997). Furthermore, recent studies examining the deletion of both Egr-1 and Egr-3 displayed a block at the DN3 stage of development, suggesting that the Egr proteins are important during β-selection but may have functionally redundant roles (Carter et al., 2007).

1.4.1.2 Positive and Negative selection

Other processes that have been extensively studied primarily through the use of transgenics are the processes of positive and negative selection. After pre-T cells have passed the β-selection checkpoint they are able to differentiate to become DP thymocytes and undergo rearrangement of the TCRα chain. Not all these rearrangements are capable of forming a functional TCR that is capable of recognising peptide-MHC; likewise some rearrangements will form a TCR which has high affinity

for peptide-MHC and thus have the potential to become autoreactive T cells in the periphery (reviewed in Starr et al., 2003; Palmer, 2003). Whilst these cells will die either by neglect and a lack of survival signals, or through the process of negative selection, the remaining cells with a medium range of affinity will receive survival signals through the process of positive selection and go on to differentiate into SP thymocytes expressing either the CD4 or CD8 co-receptor (reviewed in Basson and Zamoyska, 2000; Starr et al., 2003: Germain, 2002). The fate of these DP thymocytes depends on the TCR and the signals received and interpreted by several signalling pathways. Whilst some components of the signalling pathways for positive and negative selection are common, they diverge to achieve specific outcomes. Unlike the pre-TCR, it is interaction with peptide-MHC and the affinity of this interaction that determines the outcome for DP thymocytes. Studies with TCR ligands of subtly different affinities have demonstrated that there is a very sharp threshold of TCR-MHC affinity which determines whether a cell is positively selected or negatively selected (Daniels et al., 2006). This raises the question of how regulatory T cell development fits into this model as regulatory T cell development has been proposed to occur at a threshold of affinity in between positive and negative selection (Sakaguchi, 2004).

Efforts have also been made to visualise the process of positive and negative selection. Interestingly it was found that in response to positively selecting signals Ras was localised to the golgi, whilst in response to negatively selecting signals Ras was localised to the plasma membrane (Daniels et al., 2006). This study also showed that negatively selecting signals resulted in ERK being localised to the plasma membrane whilst in response to positively selecting signals ERK was distributed throughout the cell. Thus proposing that localisation of signalling molecules could be important in determining the outcome of the signal.

Signalling requirements for positive and negative selection have largely been investigated through the use of knockout and transgenic animals expressing dominant negatives and constitutively active mutants of components of the TCR signalsome in addition to various signalling cascades. Developmental blocks at the DP stage of thymocyte development have provided insight into the signals required for positive selection whilst the use of TCR transgenic mice such as OT-I, OT-II, F5 has also proved useful in identifying signals involved in positive selection. TCR transgenic mice on a $RAG^{-/-}$ background express a single TCR and DP thymocytes are positively selected into either the $CD4^+$ SP or $CD8^+$ SP lineage dependent on the specific TCR being expressed. While effects on negative selection have been identified by TCR

transgenic or endogenous superantigen models of clonal deletion. In the TCR transgenic models, mice express a transgenic TCR for self-peptide as well as expressing the self-peptide. In the case of the HY model (a MHC Class-I restricted TCR) the peptide is the male-antigen, thus male HY mice can be used as a model for negative selection. When negative selection is intact $CD8^+$ T cells are depleted however, a defect in negative selection results in the detection of $CD8^+$ T cells bearing the HY TCR.

The TCR is part of a signalling complex that includes CD3. Deletion of CD3δ has shown to result in a lack of ERK signalling and a block in thymocyte development (Delgado et al., 2000). Likewise deletion of protein tyrosine kinases which are recruited to the TCR such as Zap-70 results in a complete block of thymocyte development (Negishi et al., 1995). $Lck^{-/-}$ mice present a clear blockade in positive selection and Lck may also have a role in negative selection (Hashimoto et al., 1996). Again the Ras-ERK signalling pathway has been implicated downstream of the TCR. TCR activation has been shown to activate Ras (Downward et al., 1990) however, blockade of Ras resulted in defects in positive selection only (Alberola-Ila et al., 1996; Swan et al., 1995). Likewise disruption of the Ras guanine exchange factor RasGRP resulted in defects in positive selection (Dower et al., 2000) and deletion of ERK1/2 resulted in a complete lack of SP thymocytes (Fischer et al., 2005). Dominant negative forms of many of the components of the classical MAPK pathway have also revealed defects in positive selection but again not in negative selection (O'Shea et al., 1996; Alberola-Ila et al., 1995; Alberola-Ila et al., 1996). Calcium signalling through the phosphatase calcineurin has also been shown to block positive selection but not negative selection (Neilson et al., 2004). Interestingly mice deficient in the B1 subunit of calcineurin displayed a defect in ERK activation that was possibly due to the effect of calcineurin on a modulator of ERK activity.

Negative selection appears to be independent of both calcineurin and Ras-ERK signalling and is more dependent on SAPK/JNK signalling and its upstream regulators. Grb-2 is an adaptor molecule that can result in the activation of Ras through its interaction with Sos. When Grb-2 expression was reduced it was shown that JNK and p38 MAPK activation were selectively reduced but the activation of ERK was unaffected. When crossed to the HY model of negative selection, in male mice, the numbers of total thymocytes increased as well as the numbers of $CD8^+$ T cells indicating that negative selection in these mice is defective (Gong et al., 2001). Likewise a dominant negative JNK1 and inhibition of MKK6, which activates the p38

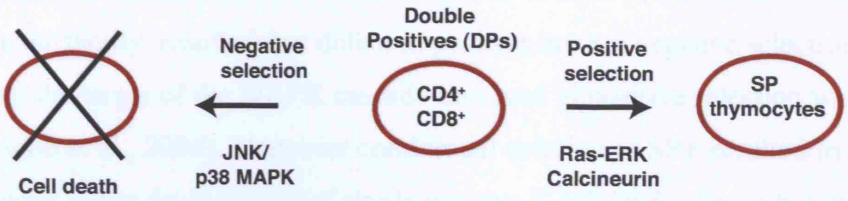


Figure 1.5. Fate of a double positive thymocyte. Double positive thymocytes can either receive signals which activate the JNK/p38 MAPK pathways which in turn lead to negative selection and cell death. Alternatively, the Ras-ERK pathway can be activated or activation of calcineruirn can occur and lead to positive selection and cell survival

MAPK, have also been shown to be selectively involved in cell death and negative selection (Rincon et al., 1998b; Sugawara et al., 1998). Recent studies have implicated another kinase in negative selection. MINK (misshapen-NCK-interacting (NIK) related kinase) was proposed to link TCR signals with JNK activation and negative selection. Using the HY model of negative selection it was shown that MINK$^{-/-}$ mice failed to delete DP thymocytes and thus demonstrated negative selection was defective in these mice (McCarty et al., 2005).

Attempts have been made to identify the transcription factors that are important in positive and negative selection (Figure 1.5). As outlined above (1.4.1.2) one of the targets of the Ras-ERK cascade are the ternary complex factor family of transcription factors and their transcription partner SRF. Deletion of SAP-1, a member of the ternary complex factor family, resulted in a defect in positive but not negative selection. This suggests that the target of the MAPK cascade involved in positive selection was the TCFs (Costello et al., 2004). Moreover conditional deletion of SRF resulted in a complete block in the development of single positive T cells indicating a block in positive selection (Fleige et al., 2007). These results correlated with observations that loss of Egr-1, a target of the TCF/SRF pathway, resulted in defective positive selection (Bettini et al., 2002) suggesting that the MAPK cascade acts via ERK and SAP-1/SRF to induce Egr-1 during the process of positive selection. Id3, a target of Egr-1 (Bain et al., 2001), was shown to be important in positive selection however, in the HY-model of negative selection Id3 deficient mice were also shown to be defective in negative selection as well as positive selection. Possibly hinting at a more profound block than the simple linear activation via Ras-ERK-Egr-1 (Rivera et al., 2000). Whilst E2A deficient animals display enhanced positive selection (Bain et al., 1997; Bain et al., 1999). Other studies investigating the requirements for negative selection have implicated the nur77 family of proteins through the use of dominant negative mutants and gene disruption. Analysis of the nur77 knockout did not display a defect in negative selection however, expression of a dominant negative did. Thus it was proposed that other members of the nur77 family were redundant with nur77 in this process (Calnan et al., 1995; Zhou et al., 1996; Lee et al., 1995).

1.4.2 αβ T cell lineage commitment to CD4$^+$ or CD8$^+$ SP thymocytes

Once αβ T cells have reached the DP stage of thymocyte development, if they express a TCR of medium affinity that will result in the T cell being positively selected, they will also undergo the process of lineage commitment into either CD4$^+$ SP thymocytes or CD8$^+$ SP thymocytes. During positive selection T cells interact with peptides presented by MHC molecules. If the T cell has interacted with MHC class I molecules the DP thymocytes will develop into a CD8$^+$SP thymocytes; on the other hand, interaction with MHC class II leads to the development of CD4$^+$SP thymocytes (reviewed in Bosselut, 2004; Singer and Bosselut, 2004; Kappes et al., 2005). Use of TCR transgenic mice, examining whether a MHC restricted TCR can be driven towards CD4$^+$ SP of CD8$^+$ SP thymocytes, has provided useful insights into the process of lineage commitment. The stochastic model proposes that the expression of CD4 or CD8 is independent of TCR specificity and that the decision is made stochastically. Since MHC recognition by a specific TCR requires the co-engagement of the correct co-receptor, this model proposes that only those T cells with the correct co-receptor will mature (Davis et al., 1993; Baron et al., 1994; reviewed in Kappes et al., 2005). Evidence for this model was provided in studies where the over-expression of a CD4 or CD8 transgene rescued the maturation of mismatched MHC:TCRs however, this rescue was weak when the transgenes were expressed at physiological levels (Baron et al., 1994; Itano et al., 1994; reviewed in Germain, 2002) . The alternative model is the instructional model which dictates that recognition and co-engagement of MHC class I with the CD8 co-receptor initiates a differentiation program which includes the down-regulation of CD4 and vice versa for MHC class II and CD4 co-engagement (Robey et al., 1991; Borgulya et al., 1991; reviewed in Kappes et al., 2005; Germain, 2002). Strong evidence exists for this model from experiments with chimeric co-receptors, where a co-receptor consisting of CD8 extracellular domain and CD4 intracellular domain was able to direct MHC class I recognising T cells into the CD4 lineage.

The instructional model dictates that the signals for lineage commitment would be driven through the co-receptors. Studies have been made to identify the signalling pathways involved. A good candidate for the critical mediator is Lck. Lck is capable of interacting with the cytoplasmic tails of both CD4 and CD8 however, in DP thymocytes it has shown to be more highly associated with CD4 (Veillette et al., 1989; Wiest et al.,

1993). Thus engagement of the CD4 co-receptor is likely to initiate a strong Lck signal whereas engagement of the CD8 co-receptor would mediate a weaker Lck signal possibly initiating the distinct differentiation programs required for lineage commitment to either $CD4^+$ or $CD8^+$ SP thymocytes. More recently the kinetic model has been proposed (reviewed in Bosselut, 2004; Kappes et al., 2005; He and Kappes, 2006), whereby all signals whether strong or weak lead to the induction of a $CD4^+CD8^{lo}$ population (Brugnera et al., 2000). T cells with TCRs recognising MHC class II molecules will still be able to signal through the co-receptor and so initiate differentiation down the CD4 lineage. T cells with TCRs that recognise MHC class I will no longer be able to signal and this lack of signal results in the CD8 differentiation program.

The Ras-ERK signalling cascade has been examined to see if it may also play a role in lineage commitment as well as positive selection. Expression of dominant negative Ras or MEK resulted in a loss of both $CD4^+$ and $CD8^+$ SP thymocytes. Likewise deletion of downstream targets such as SAP-1 and Egr-1 affected the development of $CD4^+$ and $CD8^+$ SP thymocytes equally (Costello et al., 2004; Bettini et al., 2002). However use of pharmacological inhibitors of ERK in fetal thymic organ cultures (FTOCs) resulted in a reduction in the proportion of $CD4^+$SP thymocytes, and inhibition of ERK in neonatal thymus organ culture blocked CD4 development but not CD8 development thus implicating ERK in CD4 lineage commitment (Sharp et al., 1997; Bommhardt et al., 1999). Deletion of the B1 subunit of calcineurin revealed a role for calcineurin in positive selection however there was no differential effect on either CD4 or CD8 SP thymocytes (Neilson et al., 2004). The calcineurin target gene Tox however has been shown to be involved in CD8 commitment (Aliahmad et al., 2004). The involvement of Tox was strongly associated with the up-regulation of Runx3 which has previously been shown to be important for CD4 silencing and reactivation of CD8 expression (Taniuchi et al., 2002; Ehlers et al., 2003; Sato et al., 2005). Other transcription factors which have been implicated in lineage commitment are Ets-1 - required for CD8 development (Clements et al., 2006), whilst Thpox and Gata-3 have been shown to be critical for CD4 lineage commitment (He et al., 2005; Sun et al., 2005; Pai et al., 2003; Nawijn et al., 2001). Recently the transcription factor c-Myb has also been implicated in CD4 lineage commitment, partially thorough its actions in regulating GATA-3 expression (Maurice et al., 2007). Understanding the signalling pathways which activate these transcription factors may provide insights into how the signals from the co-receptors are interpreted to direct lineage commitment as well as

possibly whether positive selection and lineage commitment are completely separate or whether there is some overlap either temporally or in the signal requirements of these processes.

1.4.3 Effector αβ T cell populations

There are many T cell subsets that come together to form a functioning immune system. In particular the $CD4^+$ T cell population is capable of further differentiation into various effector cell populations. Two well-characterised populations are the T helper 1 (Th1) and T helper 2 (Th2) T cells (reviewed in Glimcher and Murphy, 2000). Th1 cells produce high levels of IFNγ and pro-inflammatory cytokines and are important for cell mediated/inflammatory immunity. Th2 cells on the other hand produce high levels of IL-4, IL-5 and IL-13 cytokines, which are important in humoral immunity. By incubation of $CD4^+$ T cells with certain cocktails of cytokines in the presence of stimulation, it is possible to reproduce T helper differentiation *in vitro* (reviewed in Glimcher and Murphy, 2000). In particular IL-12 is able to drive Th1 differentiation whilst IL-4 is able to drive Th2 differentiation (Hsieh et al., 1993; Maggi et al., 1992; Manetti et al., 1993). In addition to cytokine driven differentiation, the signal duration through the TCR has been proposed to be important for T helper differentiation, with transient signals required for Th1 differentiation whilst prolonged signalling is required for Th2 differentiation (Iezzi et al., 1999). These results, along with studies using altered peptide ligand have led to the proposal that the affinity of the TCR may influence lineage commitment to either Th1 or Th2 (Pfeiffer et al., 1995; Kumar et al., 1995; Boyton and Altmann, 2002; and reviewed in Murray 1998).

Whilst it is possible to induce T helper differentiation *in vitro*, knowledge regarding the signals induced in this process is less clear. The role of MAPK signalling has been investigated in T helper differentiation. Experiments using mice expressing a dominant negative p38 MAPK demonstrated that p38 MAPK is required for Th1 differentiation (Rincon et al., 1998a). Corroborative evidence came from the finding that IL-12 was able to induce p38 MAPK activation (Zhang and Kaplan, 2000). JNK has been proposed to be an inhibitor of Th2 differentiation. $JNK1^{-/-}$ mice demonstrated exaggerated Th2 responses *in vitro* and in response to Leishmania infection (Dong et al., 1998; Constant et al., 2000). $JNK2^{-/-}$ mice however displayed a reduced ability to

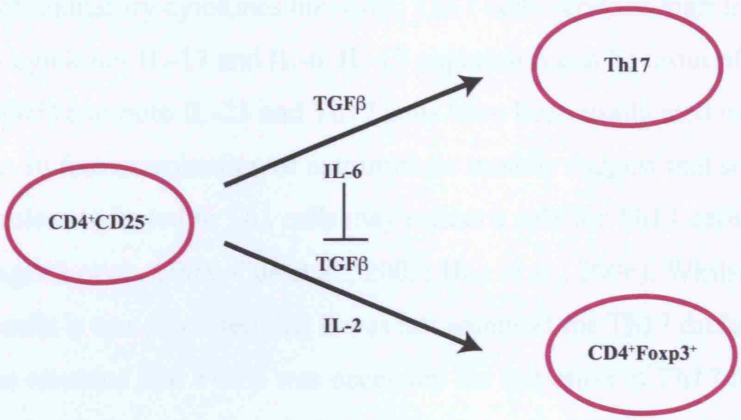


Figure 1.6. A fine balance between autoimmunity and tolerance. Naive T cells can be converted into Foxp3 expressing T cells with regulatory function through TGFβ treatment. However TGFβ plus IL-6 leads to Th17 cells which have been implicated in autoimmune disease. Thus the decision to convert to a regulatory phenotype or an autoimmune causing phenotype is a finely balanced one and would depend on the cellular context.

induce IFNγ production (Yang et al., 1998). Deletion of both JNK1 and JNK2 resembled the JNK1$^{-/-}$ mice and thus it was proposed that JNKs are important as negative regulators of Th2 differentiation (Dong et al., 2000). The ERK signalling pathway has been proposed to be important for Th2 differentiation. Mice expressing a dominant negative Ras were defective in their ability to differentiate into Th2 cells (Yamashita et al., 1999). A more recent study has proposed that ERK signalling is important for IL-4 production (Jorritsma et al., 2003).

Recently another subset of T helper cells has been described – Th17 (reviewed in Weaver et al., 2006). Th17 cells share some characteristics with Th1 cells in that they express pro-inflammatory cytokines however; Th17 cells produce high levels of the pro-inflammatory cytokines IL-17 and IL-6. IL-17 expression can be induced by IL-23 (Park et al., 2005) and both IL-23 and Th17 cells have been implicated in autoimmunity. In fact examination of autoimmune models suggest that some of the autoimmune roles attributed to Th1 cells may reflect a role for Th17 cells (Murphy et al., 2003; Langrish et al., 2005; Cua et al., 2003; Hue et al., 2006). Whilst IL-23 can induce Th17 cells it was proposed that it was not essential for Th17 differentiation, instead studies revealed that TGFβ was necessary for initiation of Th17 differentiation (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; reviewed in Veldhoen and Stockinger, 2006). Interestingly IL-6 was also shown to be required. IL-6 has been shown to inhibit regulatory T cell development, whilst TGFβ has been shown to drive differentiation of regulatory T cells *in vitro* (see section 1.5.2) (Figure 1.6). Thus when cells were cultured with TGFβ, IL-6 was shown to regulate the balance between Th17 and regulatory T cell differentiation (Bettelli et al., 2006).

1.4.4 γδ T cells

γδ T cells are a minority population within the thymus which have been ascribed roles in immune responses and the regulation of such responses (Ayadi et al., 2001; reviewed in Hayday, 2000) and thus are critical players in the maintenance of immune tolerance. T cells of this subset have been shown to be important for resistance to microbial infection as deletion resulted in an increased susceptibility to infection and/or an inability to clear the disease (Skeen and Ziegler, 1993; Rosat et al., 1993; Mombaerts et al., 1993). The role of γδ T cells in protection from infection is possibly due to the

production of IFNγ which can be induced in these cells in response to microbial antigens (Skeen and Ziegler, 1995). A study of the role of γδ T cells in murine lupus provided evidence that they were capable of both regulating and driving lupus. Depletion of γδ T cells exacerbated the disease however, deletion of αβ T cells resulted in partial development of lupus which was shown to require γδ T cell help (Peng et al., 1996). Furthermore, in synovial fluid from patients with Lyme arthritis a high proportion of γδ T cells was present which, upon stimulation, acted in a cytolytic manner towards $CD4^+$ αβ T cells (Vincent et al., 1996).

The development of γδ T cells in the thymus is little understood primarily due to a lack of cell surface markers to identify γδ precursors. Thus identifying factors important in the commitment to the γδ T cell lineage has been very difficult as most studies have only been able to look post-commitment once the cells express the γδ T cell receptor. As a general regulator of cell commitment, Notch has been shown to be important for lineage determination of αβ vs. γδ T cell lineages (reviewed in Maillard et al., 2005), whether by acting to hold a cell at a particular developmental stage until the correct differential signal is received or whether by providing the directing signals is not clear. However Notch has been proposed to act in synergy with TCR signals to promote αβ lineage development (reviewed in Hayday and Pennington, 2007).

Most γδ T cells in the periphery are double negative for CD4 and CD8 expression so it seemed likely that the γδ T cells differentiate during the DN stage of thymocyte development. Furthermore it was proposed that this was independent of TCR signalling as studies showed that high expression of IL-7R on DN2 thymocytes (i.e. prior to TCR rearrangement) biased cells towards the γδ T cell lineage (Kang et al., 2001). Identification of a γδ T cell gene profile showed that this profile was expressed in essentially all DN2 thymocytes. Coupled with the fact that rearrangement of the *Tcrg* and *Tcrd* genes, in addition to *Tcrb* genes, is initiated at this point it seems likely that γδ T cells differentiate from a common precursor during the DN2 stage of development (Pennington et al., 2003). It was also noted that the function of γδ T cells along with the expression of the γδ gene profile was defective in animals lacking a DP population such as in $TCR\beta^{-/-}$ mice (Pennington et al., 2003; Silva-Santos et al., 2005). These observations have led to the proposal that there is cross-talk between the developing αβ and γδ lineages and that the αβ T cells are capable of conditioning γδ T cells in trans. Insights from these early decisions in thymocyte development may well prove useful in understanding later developmental decisions. Upon commitment the γδ chains undergo

rearrangement and then pair to form the γδ TCR. It has been proposed that there are similar checkpoints to those in αβ T cell development. For instance it has been suggested that the adaptor protein Lat is involved in a checkpoint to ensure functional rearrangement of the γδ TCR chains (Prinz et al., 2006).

1.5 Regulatory T cells

The requirement for immunological tolerance is essential for a healthy individual; therefore there are many mechanisms that are designed to achieve this. Perhaps one of the earliest implementation of one of these mechanisms is negative selection whereby thymocytes expressing a potentially autoreactive TCR are deleted from the repertoire. As outlined above this has been proposed to be as a result of a high affinity interaction that leads to the activation of the JNK and p38 MAPK pathways and cell death. However this process is "leaky" in that even in healthy individuals autoreactive T cells can be detected in the periphery (Van Parijs and Abbas, 1998; Wekerle et al., 1996; reviewed in Sakaguchi et al., 2001). Evidence for autoreactive T cells in the periphery comes from experiments where immunisation of normal animals with self-constituents and a potent adjuvant led to autoimmune tissue damage (Weigle, 1980). It was also shown that self-reactive clones could be prepared from peripheral blood lymphocytes from healthy individuals (Wekerle et al., 1996).

A number of mechanisms exist to ensure that such autoreactive T cells are prevented from initiating an autoimmune response. These include down-regulation of the TCR and the induction of anergy. Anergy can be induced *in vitro* through TCR stimulation without co-stimulation and results in cells being unresponsive to further TCR signalling (Jenkins et al., 1987; Fields et al., 1996). There are conflicting reports as to whether ERK may be important for this process. Examination of anergic cells revealed defective signalling through Ras and ERK. However blocking ERK signalling during T cell activation, whilst capable of inhibiting proliferation in response to TCR signalling, did not result in cells becoming anergic as they were still capable of responding to TCR signalling upon re-stimulation (Fields et al., 1996; Li et al., 1996; DeSilva et al., 1998). Additionally there is a dominant mechanism of suppression that is mediated by regulatory T cells. The hypothesis that there are "suppressor" T cells was initially proposed in the 1970s; however the lack of identifying markers meant that this

hypothesis remained controversial until the identification of CD25 as a potential lineage marker (reviewed in Shevach, 2002). Many T cells have been proposed to have regulatory functions under certain conditions including γδ T cells, CD8 T cells and Gr-1^+ $CD11b^+$ myeloid suppressor cells (Peng et al., 1996; Singh et al., 2007; Mukasa et al., 1994; Song et al., 2005; Yang et al., 2006). However here I will focus on $CD4^+CD25^+Foxp3^+$ regulatory T cells (T_{reg}), sometimes also referred to as naturally occurring regulatory T cells.

1.5.1 Regulatory T cell development

1.5.1.1 Foxp3 as a lineage specific marker

The identification of a population of suppressive T cells within the $CD25^+$ T cell population by Sakaguchi and co-workers (Sakaguchi et al., 1995) renewed interest in the concept of regulatory T cells and since then the number of studies investigating regulatory T cells has expanded exponentially (reviewed in Shevach, 2000). $CD4^+CD25^+$ T cells were shown to be capable of suppressing proliferation in an *in vitro* proliferation assay (Thornton and Shevach, 1998; Takahashi et al., 1998; Piccirillo et al., 2002) and depletion of these cells from the general T cell population led to the breakdown of immunological tolerance and the development of autoimmune disease (Sakaguchi et al., 1995; Itoh et al., 1999 and reviewed in Sakaguchi, 2004; Shevach, 2000; Maloy and Powrie, 2001). The use of CD25 as a marker for regulatory T cells led to some confusion as CD25 is also a marker of activated T cells. Therefore extensive studies were performed to find other markers that were specific to the regulatory T cell lineage. Initial attempts predominantly found markers which were also up-regulated upon activation such as glucocorticoid-induced TNF-receptor family-related gene (GITR) (Shimizu et al., 2002; McHugh et al., 2002; Kwon et al., 1999; Gurney et al., 1999) and CTL-associated antigen 4 (CTLA-4) (Takahashi et al., 2000; Thompson and Allison, 1997; Bluestone, 1997).

Identification of a regulatory T cell specific marker came through studies on the scurfy mice. Scurfy mice suffer from a severe lymphoproliferative autoimmune disorder similar to the human condition IPEX (Immune dysregulation, Polyendocrinopathy,

Enteropathy X –linked syndrome). It was found that the gene responsible for this pathology was a forkhead/winged-helix transcription factor named Foxp3 (Brunkow et al., 2001). It was also shown that not only was Foxp3 expressed in regulatory T cells but it was not expressed in activated T cells and thus was a specific marker of regulatory T cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Moreover it was shown that scurfy mice had no regulatory T cells and that the lymphoproliferative disorder arose due to dysregulated proliferation of effector cells. Thus it was proposed that Foxp3 was required for regulatory T cell development (Fontenot et al., 2003; Khattri et al., 2003). This was confirmed by the findings that over-expression of Foxp3 in $CD4^+CD25^-$ T cells conferred regulatory properties onto these cells as well as up-regulating other regulatory markers (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Taken together these results led to the view that Foxp3 was necessary and sufficient for T_{reg} development and may even be the master regulator for regulatory T cells (Fontenot et al., 2005b).

However recent studies have suggested that the story is more complex (reviewed in Zheng and Rudensky, 2007). Two separate groups used expression of a tagged (GFP and EGFP) disrupted allele of Foxp3 to examine the Foxp3 developmental programme in heterozygous females where both WT and disrupted alleles can be examined in the same mouse (Gavin et al., 2007; Lin et al., 2007). It was possible to detect cells expressing either GFP or EGFP that expressed regulatory T cell markers such as GITR and CTLA-4. Although apparently committed to the regulatory lineage these cells lacked suppressive function, leading to the proposal that lineage commitment can occur without Foxp3 but that suppression requires functional Foxp3. Thus it would seem that there is a regulator of regulatory T cell development above Foxp3, although it is unclear what this may be. Recent studies have also shown that once Foxp3 expression has been initiated, it is capable of stabilising its own expression and that continued expression is required to maintain the function and phenotype of regulatory T cells (Williams and Rudensky, 2007). Therefore it would appear that "factor X" commits T cells to the regulatory T cell lineage which initiates certain developmental programs including the up-regulation of Foxp3 and that this up-regulation of Foxp3 is required to reinforce and stabilise the commitment to the T_{reg} lineage.

1.5.1.2 Selection of Regulatory T cells

Whilst the search for lineage specific markers provided some insight into regulatory T cell development, the mechanism of selection or differentiation into this lineage and the signalling pathways that may be involved are not fully understood. Experiments using day 3 thymectomys showed that regulatory T cells developed within the thymus and it was possible to detect these cells 3 days after birth with levels expanding to near adult levels over the following two weeks (Asano et al., 1996). T_{reg} development was shown to require interactions between TCR and MHC molecules (Bensinger et al., 2001). Furthermore the ability to induce autoimmunity through the depletion of $CD25^+$ cells from the $CD4^+$ SP T cell population has led to the view that regulatory T cells differentiate from $CD4^+$ SP thymocytes post positive selection (Modigliani et al., 1996; reviewed in Sakaguchi, 2000). Experiments with TCR transgenic mice also demonstrated that the development of regulatory T cells can be detected as early as the DP stage of thymocyte development (Cabarrocas et al., 2006).

It has been proposed that T_{regs} are selected on a high affinity TCR:MHC interaction. Evidence for this idea came from the finding that mice expressing a transgenic TCR on a $RAG^{-/-}$ background did not have regulatory T cells or they were only present in extremely small numbers (Hori et al., 2002; Itoh et al., 1999; Jordan et al., 2001). When transgenic TCRs are expressed on a RAG WT background it was found that the majority of T_{regs} used endogenous α chains to form the TCR and thus it was proposed that due to the requirement of a high affinity interaction for the selection of T_{regs}, only those which used endogenous α chains could be selected into the regulatory T cell lineage. Interestingly, in one model the T_{regs} that did express both the α and β chains of the transgenic TCR also appeared to express a second endogenous α chain (Hori et al., 2002). Furthermore experiments in which mice expressing a transgenic TCR were crossed with mice expressing the cognate antigen resulted in an increased proportion of thymocytes developing into regulatory T cells in the double transgenic mice. These data again suggested that T_{regs} might be selected on high affinity interactions (Jordan et al., 2001; Apostolou et al., 2002). It is not clear whether this is also a high affinity interaction similar to those which lead to negative selection or whether it may be modulated by as yet unknown factors which may initiate the T_{reg} lineage programme.

It has also been suggested that the level of peptide expression as well as the nature of this presentation may affect the efficiency of T_{reg} development (Kawahata et al., 2002; Lerman et al., 2004; Pacholczyk et al., 2002). The increased proportion of regulatory T cells seen in the TCR transgenic mice crossed to mice expressing the cognate peptide did not always correlate with substantially increased numbers of regulatory T cells (van Santen et al., 2004) which some have interpreted to indicate that T_{regs} are not selected on a high affinity interaction. However there are other models that may explain these apparently contradictory results. Studies on the development of γδ T cells have provided interesting insights in thymocytes development suggesting that thymocyte development is not a simple linear development programme. It was shown that the later stages of development are capable of influencing early development via a process of trans conditioning (Pennington et al., 2006). These studies led to the proposal that T_{reg} cells may be generated from a distinct DP thymocyte population which develops from non-conditioned DN2 thymocytes (Pennington et al., 2006). These cells undergo a lower expansion phase than the majority of DP cells and consequently diversion into this lineage may not result in a large increase in absolute numbers of regulatory T cells.

The idea that regulatory T cells suppress the activity of self-reactive T cells coupled with the view that these cells are selected on the basis of a high affinity interaction led to the investigation of whether regulatory T cells were themselves self-reactive. Studies looking at TCR diversity have shown that the repertoire of T_{regs} is distinct from that observed on non-regulatory T cells (Pacholczyk et al., 2006), whilst studies looking at the CDR3 region of the TCR chains also showed a distinct repertoire for T_{regs} (Hsieh et al., 2004; Hsieh et al., 2006). The CDR3 region of the TCR chains is one of the areas of highest diversity and thus strongly influences the specificity of the TCR. Examination of the CDR3 regions in $CD4^+CD25^+$ and $CD4^+CD25^-$ populations with a limited TCR repertoire revealed equivalent diversity in the sequences within the two populations but with little overlap between the two. Moreover, through the transgenic expression of these particular sequences it was shown that the $CD4^+CD25^+$ TCR sequences were capable of initiating substantial expansion in a lymphopenic environment, whereas sequences taken from $CD4^+CD25^-$ T cells did not. Thus it was proposed that regulatory T cells were self-reactive (Hsieh et al., 2004). Further validation of this result came from examination of the $CD4^+CD25^+$ T cell population in scurfy mice. These cells are self-reactive cells that have become activated due to the lack of regulatory T cells in these animals. Interestingly the CDR3 sequences of these

cells overlapped with those found in WT regulatory T cells whilst naïve $CD4^+CD25^-$ T cells were distinct, demonstrating that regulatory T cells present TCRs which are self-reactive (Hsieh et al., 2006).

1.5.1.3 TCR signalling pathways involved in T_{reg} selection

Little direct evidence has addressed the role of signalling pathways involved in T_{reg} development. Therefore our understanding of how a high affinity interaction may lead to the up-regulation of Foxp3 and regulatory T cell development is poor. Evidence for many of the factors proposed to be involved in T_{reg} development has come from mice with autoimmune phenotypes, although these could quite easily be factors important for function rather than specifically development.

SHP-1

SHP-1 is a negative regulator of TCR signal. Moth eaten mice which lack SHP-1 expression display increased amounts of $CD4^+CD25^+$ T cells (Carter et al., 2005). Whilst this supports the idea that TCR signals promote T_{reg} development, it is unclear which proteins may act as mediators of this signal.

RasGRP

Another molecule which has been suggested to be involved in T_{reg} development is RasGRP, an activator of Ras signalling upon TCR ligation (Ebinu et al., 2000). $RasGRP1^{-/-}$ mice develop autoimmunity and this observation led to the suggestion that RasGRP may be important for tolerance (Layer et al., 2003). Disruption of RasGRP has been shown to result in a block in positive selection. Whilst the few mature SP T cells that did develop displayed an activated phenotype, the development of regulatory T cells was not examined. Therefore it is not clear whether the autoimmunity that arises in these animals is due to a block in T_{reg} development or whether the activated T cells are dysregulated.

LAT

Further insight has been gained from mice with a mutation in the transmembrane adaptor protein linker for activation of T cells (LAT). LAT is part of the signalling complex which meditates TCR activation (Zhang et al., 1998) and disruption was

shown to block positive selection of mature thymocytes (Zhang et al., 1999). In a more recent study however a specific mutation that blocked LAT interaction with phospholipase C (PLC)-γ was employed. These mice develop autoimmunity which would appear to be due to a block in T_{reg} development (Koonpaew et al., 2006), thus linking TCR signals to T_{reg} development. Whilst the involvement of RasGRP needs further investigation, it is interesting that the LAT mutation that specifically blocked T_{reg} development was defective in its ability to bind PLC-γ. As RasGRP has been proposed to link TCR activation, PLCγ and the Ras-ERK signalling pathway, RasGRP may indeed have a role in regulatory T cell development that in turn may implicate the Ras-ERK signalling pathway.

1.5.1.4 Cytokines and other co-factors involved in T_{reg} development

There is increasing evidence that TCR signalling alone may not be sufficient to direct T_{reg} development particularly from experiments with TCR transgenic mice crossed onto mice expressing cognate peptide. Whilst the proportion of T_{regs} is substantially increased, this "conversion" is only ever partial which has led to the idea of a limiting factor during T_{reg} development and the idea that regulatory T cell development requires two signals (reviewed in Liston and Rudensky, 2007). In particular, cytokines have been proposed as a possible source of this second signal.

IL-2

The role of IL-2 in regulatory T cell development has also been extensively studied although sometimes with conflicting views. IL-2Rα, IL2-Rβ and IL-2 deficient mice display an autoimmune phenotype (Kramer et al., 1995; Almeida et al., 2002; Suzuki et al., 1999). However further analysis demonstrated the presence of regulatory T cells albeit at a reduced number in the IL-2 and IL2Rα deficient mice although the loss of the IL-2 common γ chain did result in a complete loss of $Foxp3^+$ T cells (Fontenot et al., 2005a). According to this model, IL-2 signalling functions in the survival of regulatory T cells and is required for their homeostasis in both the thymus and periphery (Fontenot et al., 2005a).

TGFβ

Another cytokine that has been investigated for a potential role in T_{reg} development is TGFβ. TGFβ1$^{-/-}$ mice exhibit embryonic lethality to varying degrees depending on the genetic background however, those mice which are born develop a severe lymphoproliferative disease and die shortly after birth (Kulkarni et al., 1993; Shull et al., 1992). Dissection of the mechanism of this autoimmunity is complicated by the multitude of effects that has been reported for TGFβ. However various transgenic and conditional deletions have provided insights which suggest that whilst TGFβ is required for the suppressive effects of regulatory T cells, it is not required for the development of these cells (Marie et al., 2006; Marie et al., 2005; Li et al., 2006).

CD28

The co-stimulatory molecule CD28 has also been proposed to be important for the development of regulatory T cells. Initially experiments looking at CD4$^+$CD25$^+$ T cells proposed that CD28 was important in the maintenance of T_{regs} in the periphery. Later analysis with the Foxp3 marker however, showed that the number of thymic CD4$^+$Foxp3$^+$ T cells was also reduced in CD28 deficient T cells (Salomon et al., 2000; Tang et al., 2003; Sansom and Walker, 2006; Tai et al., 2005). It was proposed that the loss of CD28 resulted in reduced T_{regs} through two mechanisms; (i) IL-2 levels were reduced to a level that was insufficient for T_{reg} homeostasis and (ii) an additional undefined cell intrinsic mechanism (Tai et al., 2005). Thus the apparent requirements for IL-2 and CD28 in regulatory T cell development has led to another model for T_{reg} development whereby development occurs as a two-step process (possibly akin to lineage commitment of CD4$^+$ and CD8$^+$ SP thymocytes – see section 1.4.2). The first step would involve the high affinity interaction between the TCR and MHC:self-peptide which primes the cells to receive additional signals, possibly involving a currently unknown regulator of T_{reg} commitment. The second step then involves secondary signals possibly from IL-2 or other unknown cofactors that then initiates Foxp3 expression and development of regulatory capacity (reviewed in Liston and Rudensky, 2007).

1.5.2 Extrathymic generation of Regulatory T cells

For many years it has been possible to generate a toleragenic response by repeated administering of low dose antigen (reviewed in Lohr et al., 2006). Although the mechanisms of this induced tolerance are unclear, it appears to result in the extrathymic generation of regulatory T cells usually either Tr1 or Th3 cells which express high levels of the suppressive cytokines IL-10 or TGFβ (reviewed in Lohr et al., 2006). More recently it has been shown that peripheral $CD4^+CD25^-Foxp3^-$ T cells can be converted into $Foxp3^+$ expressing cells with regulatory properties. Whilst this may have therapeutic potential, much controversy surrounds this process. *In vitro* experiments have shown that by activating naïve T cells in the presence of TGFβ Foxp3 expression can be induced (Chen et al., 2003; Fantini et al., 2004). Whilst it is not known how TGFβ specifically induces Foxp3 expression, it has been shown that IL-2 is required for this process (Zheng et al., 2007; Davidson et al., 2007). Thus *in vitro* peripheral conversion appears to have similar requirements to naturally occurring T_{regs}, which require IL-2 for development and TGFβ for the maintenance of Foxp3 expression. However part of the controversy surrounding this process is whether it is physiologically relevant. It has been suggested that the amount of TGFβ used in these assays is too high to ever occur *in vivo*. There is also conflicting data regarding the role of TGFβ induced T_{regs} *in vivo*, with some studies demonstrating that TGFβ can induce T_{regs} whilst other suggest this is not the case (Kretschmer et al., 2005; Coombes et al., 2007; Wong et al., 2007). Many more extensive studies are required to confirm whether this is indeed a physiological mechanism. For instance it is not clear whether the up-regulation of Foxp3 is a sustained or transitory increase. Interestingly the induction of Foxp3 expression by TGFβ can be inhibited by IL-6. This combination of cytokines in coupled with TCR signalling has been proposed to initiate the development of Th17 cells which have been implicated in a number of autoimmune responses (reviewed in Weaver et al., 2006). Thus emphasising that the balance between autoimmunity and tolerance can be a very fine one.

1.5.3 Regulatory T cell function

As the name suggests regulatory T cells are important in regulating the immune system. They have been implicated in a number of processes and were initially identified on the basis of preventing autoimmune disease. However they have also been implicated in regulating immune responses in particular during Leishmania infection. The persistence of Leishmania in mice, which is required for effective memory and resistance to re-infection, was shown to require regulatory T cells (Belkaid et al., 2002). Upon depletion of regulatory T cells Leishmania was completely cleared from infected mice and so upon re-exposure to infection these mice were susceptible to re-infection. Mice which had not had T_{regs} depleted were able to prevent significant disease from establishing upon re-exposure. It has also been proposed that regulatory T cells are part of the mechanism by which tumour cells avoid generating an immune response (Shevach, 2004). Experiments where $CD4^+CD25^+$ T cells were depleted showed that they were responsible for inhibiting anti-tumour responses (Shimizu et al., 1999). Thus an understanding of not only the development of these cells but their function could have important physiological relevance.

The requirements for these cells to function correctly are not well understood. Different models of suppression have been shown to involve different factors. $CD4^+CD25^+$ T cells were initially shown to be capable of suppressing the proliferation of $CD4^+CD25^-$ T cells *in vitro* in a cytokine independent contact dependent manner (Takahashi et al., 1998; Thornton and Shevach, 1998). However regulatory T cells have also been shown to be important in many models of autoimmunity where their ability to suppress the immune response has been shown to additionally require suppressive cytokines such as IL-10 and TGFβ. Different models have shown different dependency on suppressive cytokines for instance, the *in vivo* model of colitis and inflammatory bowel disease (IBD) has shown that the suppression of colitis requires both IL-10 and TGFβ (Asseman et al., 1999; Powrie et al., 1996), whilst suppression of autoimmune thyroiditis in the rat requires IL-4 and TGFβ (Seddon and Mason, 1999).

1.5.3.1 Mechanisms of Suppression

It has been demonstrated that regulatory T cells suppress proliferation and IL-2 production by effector T cells *in vitro* (Thornton and Shevach, 1998; Takahashi et al., 1998; Piccirillo et al., 2002). Furthermore these studies demonstrated that TCR activation was required for this process. In addition to blocking proliferation, recent studies have examined the possibility that T_{regs} may induce apoptosis of effector cells. Analysis of human T_{regs} showed that granzyme A was expressed in these cells and that they were capable of inducing apoptosis in their target cells (Grossman et al., 2004a; Grossman et al., 2004b). Similarly, murine T_{regs} were capable of preventing allograft rejection through apoptosis of the $CD8^+$ memory cells which mediated the allograft rejection (Dai et al., 2004). Furthermore, granzyme B was identified as being down-regulated in response to anti-GITR mediated inhibition of suppression and T_{regs} from granzyme $B^{-/-}$ mice displayed a reduced capacity for *in vitro* suppression (Gondek et al., 2005). However perforin deficient T_{regs} were fully functional. Finally, it was shown that suppression by WT T_{regs} in an *in vitro* suppression assay involved the induction of apoptosis of effector cells simultaneously with a reduction in proliferation (Gondek et al., 2005).

1.5.3.2 Effector resistance to suppression

In addition to identifying molecules that are important in mediating suppression, there is also an increasing understanding of molecules that can induce effector cells to become resistant to T_{reg} suppression. There is some evidence that GITR may act in this manner (Stephens et al., 2004; Tone et al., 2003), although there is also evidence that it acts directly on the regulatory T cells. The E3 ubiquitin ligase Cbl-b has been shown to regulate responsiveness to TGFβ mediated suppression (Wohlfert et al., 2006). There are certain occasions when suppression by regulatory T cells could be detrimental, either in the control of a pathogenic response or the suppression of anti-tumour immunity. Therefore there appear to be mechanisms for preventing immune suppression by regulatory T cells. In particular it has been shown that activation of dendritic cells (DCs) by toll-like receptors (TLRs) can reverse T_{reg} suppression (Pasare and Medzhitov,

2003; Fehervari and Sakaguchi, 2004). Again the mechanism of this resistance is not fully understood but has been proposed to involve co-stimulation and IL-6.

1.5.3.3 Do T_{regs} act directly on effector cells?

Despite the increasing knowledge with regards to molecules involved in T_{reg} suppression as well as those capable of blocking T_{reg} suppression, it is still not clear whether T_{regs} interact directly with effector cells or whether they act via DCs. The most likely scenario is that both situations can occur dependent on the environmental context. APC deficient *in vitro* suppression assays have shown that $CD4^+CD25^+$ T cells are capable of direct suppression of effectors (Shevach, 2002). However $CD4^+CD25^+$ T cells have also been shown to down-regulate co-stimulatory molecules on immature dendritic cells (Cederbom et al., 2000).

1.5.3.4 Regulatory T cell signalling

Regulatory T cells have been shown to be anergic in response to stimulus which would normally induce the proliferation of conventional $CD4^+$ T cells (Thornton and Shevach, 1998; Takahashi et al., 1998), although they have been shown to be capable of proliferation *in vivo* (Walker et al., 2003). Thus the anergic phenotype may simply be as a result of having different second signalling requirements to conventional T cells. In agreement with this, T_{regs} can be induced to proliferate *in vitro* by the addition of exogenous IL-2, although this was also reported to abrogate their regulatory function when added during an *in vitro* suppression assay (Thornton and Shevach, 1998; Takahashi et al., 1998). Studies have shown that activation of T_{regs} through the TCR results in a lower activation of many classical signalling pathways known to be downstream of the TCR in conventional T cells (Hickman et al., 2006; Tsang et al., 2006). Despite these studies using different cells, murine in one study and human in the other, calcium and ERK signalling were shown to be reduced when compared with that observed in conventional T cells. Moreover, enhancement of PKC and Ras signalling pathways resulted in the proliferation of regulatory T cells. These data suggest that T_{regs} are not globally unresponsive but are conditioned to quench signals that would normally

induce proliferation. However few other studies have investigated the signals that lie downstream of TCR activation and which may be required for efficient T_{reg} function.

1.5.3.5 Mediators of contact dependent mechanism of suppression

Whilst the signalling pathways responsible for T_{reg} suppression have not been extensively studied, efforts have been made to identify potential surface mediators of the contact dependent mechanisms observed in both *in vitro* and *in vivo* models of suppression. Insights into the molecules directly responsible for contact dependent suppression may in future reveal the signalling pathways involved. Some confusion has arisen about the relative involvement of some molecules, possibly due to differences between *in vivo* and *in vitro* models.

CTLA-4

CTLA-4 deficient mice suffer from a severe lymphoproliferative disorder and many die within a few weeks of birth similar to that observed for the scurfy mice (Waterhouse et al., 1995; Tivol et al., 1995). CTLA-4 is a CD28 homologue which interacts with its ligand B7 and has shown to be inhibitory rather than co-stimulatory (Thompson and Allison, 1997; Bluestone, 1997). Thus the development of the severe lymphoproliferative disorder in these mice has been proposed to be due to a lack of inhibitory signals during T cell activation. CTLA-4 was shown to be highly expressed on T_{regs} and was shown to be required for T_{reg} control of intestinal inflammation (Read et al., 2000). However CTLA-$4^{-/-}$ mice do generate regulatory T cells that are capable of mediating suppression *in vitro* albeit a reduced level of suppression when compared to WT T_{regs} (Takahashi et al., 2000). However blocking CTLA-4 on WT T_{regs} abrogated T_{reg} mediated suppression *in vitro* (Tang et al., 2004). Clarification of a possible role for CTLA-4 in T_{reg} suppression came from experiments where TGFβ signalling was inhibited. These experiments demonstrated that CTLA-$4^{-/-}$ T_{reg} mediated suppression was TGFβ dependent in contrast to the cytokine independent mechanism used by WT T_{regs} (Tang et al., 2004). Thus CTLA-4 would appear to be important for T_{reg} suppression. Recent studies suggest that CTLA-4 may be central to the suppression of IL-2 transcription in effector cells that occurs during *in vitro* suppression assays. *In vitro* suppression assays initiate the expression of the transcriptional repressor ICER which correlates with a reduction in the number of cells producing IL-2 (Bodor et al.,

2007). CTLA-4 blockade inhibits the induction of ICER, and B7 deficient effectors were unable to induce ICER. These data suggest that CTLA-4 is required for the induction of ICER and subsequent repression of IL-2 in effector cells during *in vitro* suppression assays. Interestingly enforced Foxp3 expression also increased the expression of ICER suggesting that in regulatory T cells ICER may be highly expressed. Perhaps ICER could account for the low proliferative capabilities and failure to induce IL-2 expression seen in T_{regs} *in vitro*.

GITR

Another molecule that is highly expressed on regulatory T cells is GITR. GITR is a member of the tumour necrosis family and recently its ligand has been identified and found to be expressed on a variety of cells including APCs (Tone et al., 2003). It is unclear whether GITR has a role in suppression. Signalling through GITR has been shown to activate ERK and is a co-stimulatory molecule for most T cells including regulatory T cells (Ronchetti et al., 2004; Tone et al., 2003). $GITR^{-/-}$ T_{regs} do develop and are suppressive (Ronchetti et al., 2004; Tone et al., 2003). However addition of an agonistic anti-GITR antibody blocked suppression in an *in vitro* suppression assay (McHugh et al., 2002; Shimizu et al., 2002) and in these experiments it was proposed that the action of GITR was to inhibit T_{reg} function directly. However other experiments have proposed that GITR acts to make effector cells resistant to suppression (Stephens et al., 2004; Tone et al., 2003). Thus further studies are required to clarify the action of GITR and it may well prove to be that the action of GITR will depend on other environmental factors.

CD103

Another marker that has been shown to be expressed on a subset of T_{regs} is CD103, also known as $\alpha_E\beta_7$ integrin. CD103 is expressed on approximately 20% of regulatory T cells and at present does not appear to be required for contact dependent suppression. Nonetheless it has been proposed to identify T_{regs} with a greater suppressive capability (Lehmann et al., 2002; McHugh et al., 2002; Banz et al., 2003). Examination of regulatory T cells expressing CD103 displayed an effector/memory like phenotype (Huehn et al., 2004). Furthermore it was proposed that naïve T_{regs} ($CD4^+CD25^+CD103^-$) preferentially re-circulated through lymph nodes whilst the effector/memory T_{regs} ($CD4^+CD25^+CD103^+$ and $CD4^+CD25^-CD103^+$) represent

inflammation seeking T_{regs} and where shown to be able to suppress active inflammation in an antigen-induced model of arthritis (Huehn et al., 2004).

LAG-3

Lymphocyte activating gene 3 (LAG-3), a CD4-related molecule, has recently been shown to be highly expressed on induced regulatory T cells (Huang et al., 2004). How this protein may regulate suppression is not clear and it is interesting that its expression is only present on the surface of regulatory T cells after they have been activated. Activation of T_{regs} with IL-2 prior to incubation with effector cells has been shown to increase the suppressive capabilities of regulatory T cells (Thornton et al., 2004), perhaps this may be mediated by an increased expression of LAG-3 on the cell surface. The addition of LAG-3 antibodies was found to block *in vitro* suppression suggesting that this molecule is important in T_{reg} mediated suppression. As with Foxp3, ectopic expression of LAG-3 was sufficient to confer regulatory properties on non-regulatory T cells. Furthermore in *in vitro* suppression assays where the effector T cells were activated with antigen, LAG-3 deficient regulatory T cells displayed reduced suppressive capabilities at high antigen dose when compared with WT T_{regs} (Huang et al., 2004). Once again further studies are required to link these single observations into a mechanism for suppression.

TGFβ

$TGF\beta^{-/-}$ mice develop a severe lymphoproliferative syndrome similar to $Foxp3^{-/-}$ animals (Shull et al., 1992; Fontenot et al., 2003). TGFβ has been implicated in the regulation of autoimmunity in various models (reviewed in Letterio and Roberts, 1998). Despite the autoimmune syndrome in $TGF\beta^{-/-}$ mice, examination of neonatal T_{reg} populations found no difference in the numbers or function of regulatory T cells in $TGF\beta^{-/-}$ mice (Piccirillo et al., 2002). Studies using the *in vivo* model of colitis have previously demonstrated a role for TGFβ in regulatory T cell mediated suppression although it is not clear if the TGFβ is produced by the T_{regs} (Powrie et al., 1996). Interestingly mice expressing a dominant negative TGFβ receptor type II, and thus unable to respond to TGFβ signalling, generated effectors that appeared to be resistant to T_{reg} mediated suppression in the colitis model, suggesting that *in vivo* TGFβ is important for T_{reg} mediated suppression (Fahlen et al., 2005).

It has been proposed that that TGFβ may act in *in vitro* suppression assays by binding to receptors on the T_{reg} cell membrane and acting in a cell contact manner (Nakamura et al., 2001). However other groups suggest that there is no requirement for TGFβ in *in vitro* suppression assays (Piccirillo et al., 2002). Generally however, the data suggest that TGFβ does have a role in T_{reg} mediated suppression particularly *in vivo*. TGFβ appears to be able to act in T_{reg} mediated suppression as a compensatory mechanism when certain aspects of the normal mechanism of suppression are inhibited as in the case of CTLA-$4^{-/-}$ regulatory T cells (see above). Thus it would seem that TGFβ is likely to have a role in T_{reg} suppression although the dissection of the exact nature may be difficult. Recent studies have proposed that TGFβ is important for the homeostasis of regulatory T cells by promoting the expression of Foxp3 as well as regulating the size of the peripheral compartment of T_{regs} (Marie et al., 2005; Schramm et al., 2004). Such a role would be consistent with the ability of TGFβ to induce *de novo* expression of Foxp3 in naïve $CD4^{+}CD25^{-}$ T cells *in vitro*.

IL-2

It has been proposed that T_{regs} may simply sequester IL-2 due to the high expression of the IL-2R on their surface and thus the reduced proliferation in *in vitro* suppression assays is as a result of IL-2 being a limiting factor. However this would not explain the repression of IL-2 transcription in effector cells which has been described. Recent *in vitro* studies have shown that IL-2 is required for the function of regulatory T cells and that pre-activation of regulatory T cells in culture medium containing IL-2 can increase the suppressive functions of regulatory T cells (Thornton et al., 2004).

Whilst our understanding of potential mediators in T_{reg} mediated suppression has increased from these studies, these studies have also highlighted the differences between *in vitro* and *in vivo* function.

1.6 Aim of this thesis

The Ternary Complex Factors as targets of the MAPK signalling cascades have the potential to be involved in many cellular processes. In the immune system MAPK cascades have been shown to be involved in thymocyte development and effector cell differentiation and function. The TCFs were shown to be expressed in total thymocytes and removal of the predominant TCF in thymocytes SAP-1 resulted in a severe defect in

positive selection (Costello et al., 2004). Despite the other TCF knockouts failing to demonstrate an immunological phenotype (Cesari et al., 2004; Ayadi et al., 2001) it remains possible that specific immune functions which have not been examined are defective in these animals. Moreover it is possible that the TCFs have redundant roles within the immune system and as such functions for the TCFs may only be revealed upon depletion of multiple TCFs. As such analysis of TCF deficient animal models represents an opportunity to further our understanding of various developmental and functional aspects of the immune system.

Whilst positive and negative selection during thymocyte development have been extensively studied with regards to their mechanism and signalling requirements, the requirements for regulatory T cell development have not been as extensively studied. There are now various proposed models for T_{reg} development including 1) development from cells that have been positively selected, 2) rescue from negative selection through the expression of Foxp3, 3) development from a subset of DP thymocytes which themselves have arisen from a non-conditioned population of DN2 thymocytes and 4) a two step model incorporating selection on a high affinity interaction between MHC and TCR which commits to the T_{reg} lineage followed by a second signal which initiates the T_{reg} development programme. Insights into the signalling pathways involved in T_{reg} development and/or regulating Foxp3 expression may provide a better understanding as to which of the above models are correct or partially correct. For instance the distinction as to whether these cells undergo positive or negative selection could be examined by determining which of the MAPK cascades are activated during T_{reg} development. PLCγ activation has been proposed as a mediator of T_{reg} development and PLCγ has previously been shown to activate the ERK signalling pathway. Interestingly recent studies have also implicated the Ras GEF RasGRP in maintaining tolerance (Layer et al., 2003). Thus these results suggest that the Ras-ERK signalling pathway may be important for regulatory T cell development.

To examine this further I have used SAP-$1^{-/-}$ animals to investigate T_{reg} development and function. In addition I have examined the roles of the other TCF family members, either singly or as multiply deficient animals in T_{reg} development to assess the possibility of the TCFs having specific roles in T_{reg} development or alternatively being redundant in this process. Furthermore, the potential role for the TCF transcription partner SRF in regulatory T cell development was also examined. Finally I have used mice expressing a dominant negative Raf transgene to investigate the importance of the Ras-ERK signalling pathway in T_{reg} development and function.

2 Material and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

This is a general list of reagents used. Reagents used for specific experiments will be detailed in the relevant section. Unless stated otherwise chemicals were obtained from Sigma.

^{3}H Tritium	Amersham
^{32}P dCTP	Amersham
40% Acrylamide/Bisacrylamide 37.5:1	AMRESCO
Agarose	Gibco BRL
Ammonium persulphate	Sigma
Boric Acid	Fischer Scientific
Bromophenol Blue	Biorad
Dithiothreitol (DTT)	Calbiochem
Ethidium Bromide	Boehringer Mannheim
β-Mercaptoethanol	Sigma
Milk powder	Marvel
dNTPs	Pharmacia
Okadaic acid	Calbiochem
Orange G	Sigma
Poly(dIdC)·poly(dIdC)	Amersham
Spermidine	Sigma
TEMED	Sigma

Xylene cyanol	Biorad
3MM paper	Whatman

2.1.2 Buffers and Solutions

All buffers and solutions were made with deionised water (Milli-Q plus system, Millipore) and where appropriate sterilised by filtration on a 0.2 μm vacuum-driven filtration system (Stericup).

PBS	0.17mM NaCL 3 mM KCl 1mM Na_2HPO_4 1.8mM KH_2PO_4 pH 7.4
TBE	89mM Tris Base 89mM Boric Acid 2mM EDTA
TEN	10mM Tris pH 7.5 1mM EDTA pH7.5 100mM NaCl
Saponin Buffer	Saponin PBS 10% Fetal Calf Serum (PAA laboratories) 10mM HEPES pH 7.4
Hypotonic lysis buffer	dH_20 155mM NH_4Cl 1mM $KHCO_3$ 10μM EDTA
FACs Buffer	PBS 1% Heat inactivated Fetal Calf Serum (FCS)
RPMI cell culture media	RPMI-1640 (produced by CRUK Research Services)

RPMI cell culture media cont.	10% Heat Inactivated Fetal Calf serum 50μM β-Mercaptoethanol
Freezing media	5% DMSO (Fischer Scientific) 95% Heat Inactivated Fetal Calf Serum
Bandshift cell extraction buffer	20mM HEPES pH 7.9 3mM Spermidine 10% glycerol 0.4M NaCl 0.4% Triton X-100 10mM EGTA, 5mM EDTA 1mM dithiothreitol 1μg/ml okadaic acid Protease inhibitors
Bandshift SRF core dilution buffer	100mM NaCL 20mM Tris 1mM DTT protease inhibitors
Bandshift Probe Loading buffer	60% Glycerol 10mM EDTA 0.01% Xylene cyanol 0.01% Bromophenol blue
Bandshift Probe extraction buffer	0.5M NH_4OAc 1mM EDTA
Bandshift reaction buffer	10mM Tris-HCl pH 7.9 50mM NaCL 1mM EDTA 3mM dithiothreitol 50ng/ml ovalbumin 50ng/ml poly(dIdC)·poly(dIdC), 10% Ficoll 400 Protease inhibitors

Bandshift reaction buffer cont.	0.01% Xylene cyanol 0.01% Bromophenol blue
Western Sample Buffer	50mM Tris-HCl pH6.8 100mM dithiothreitol 2% SDS 0.1% Bromophenol blue 10% glycerol
Western Running Buffer	25mM Tris 0.4M Glycine 0.1% SDS
Western Transfer Buffer	dH_20 20mM Tris 0.2M Glycine 10% Methanol
Western Blocking Buffer	PBS 0.2% Tween 5% Milk
Western Wash Buffer	PBS 0.2% Tween
Western Antibody Buffer	PBS 0.2% Tween 2% Milk
Western Stripping Buffer	dH_20 0.1M Glycine pH2.5 0.1% SDS
Agarose-DNA loading buffer	80% Glycerol 50mM EDTA 0.1% Orange G

2.2 Methods

2.2.1 Mice

SAP-1$^{-/-}$ mice and mice expressing a dominant negative Raf transgene have previously been described (Costello et al., 2004 and O'Shea et al 1996.). Elk-1$^{-/-}$ mice and Netδ mice have previously been described (Cesari et al., 2004; Ayadi et al., 2001). Double TCF deficient and triple TCF deficient animals were generated in collaboration with A. Nordheim and B. Wyslek. SRF flox mice have been previously described (Parlakian et al., 2004). CD2 Cre mice were obtained from D. Kioussis. Mice were maintained in specific pathogen-free conditions in the Cancer Research UK Biological Resources Unit. Animal experimentation was approved by the Cancer Research UK Research Services Animal Ethics committee.

2.2.2 Genotyping

Tail or ear snips were taken and DNA extracted using DNA extraction kit (Qiagen) in either the 96 format or spin column format according to the manufacturer's instructions with the additional step of heating lysed snips at 55°C for 15 min prior to the start of the protocol. Genotyping was performed using the following primers and conditions:

Gene	5' Primer	3' Primer
SAP	GTGATGATGGTGGGATAGTGTAT ATCTTGCAGGAGTG	CCAGAGCCACTGAAGCTGTTAAGTA AACTAGACTAAAC
Elk-1 WT	GGCTATGGGGACTGCGCAAGAA CAAGACC	GCCATAAACTACCGAGGACAGAAA GCACAGG
Elk-1 KO	GCCCCTCCCCCGTGCCTTCCTT	TAGCAGCCACAACCAATCCATTTCT CCATC
NET	TGAAACGTGTAATCCTTGTGTCC TC	TAATTTCCAAGTTCTGGGCACGTAG
RAF	ATGGAGCACATACAGGGAGCTT GG	CCTCCTCCGATATGAAGTTTCTGTTC

	Program 1	
Step	Temperature	Time
1	95°C	3 min
2	95°C	30 secs
3	64°C	1 min
4	72°C	1 min
5	72°C	10 min
35 Cycles		

	Program 2	
Step	Temperature	Time
1	95°C	3 min
2	95°C	30 secs
3	69°C	1 min
4	72°C	1 min
5	72°C	10 min
33 Cycles		

	Program 3	
Step	Temperature	Time
1	95°C	3 min
2	95°C	30 secs
3	60°C	45 secs
4	72°C	1 min
5	72°C	10 min
30 Cycles		

Elk-1 WT, Elk-1 KO, Net were run on program 1, SAP was run on program 2, Raf run on program 3. SAP-1 and Elk-1 primers and PCR protocol designed by Rob Nicolas, Net primers and PCR protocol as published (Ayadi et al., 2001), DN Raf as published (O'Shea et al., 1996). PCR reactions were made to a final volume of 40µl. Biotaq Red Taq polymerase and buffers were purchased from Bioline. PCR products were analysed on 2% agarose gels run in TBE at 120V and subjected to UV light.

2% agarose gel	2% w/v agarose TBE Ethidium Bromide

OT-II TCR transgenic mice were typed for expression of the transgenic TCR via tail bleeds. Tail bleeds were collected and a 30μl aliquot was removed, red blood cells were lysed by addition of 500μl of hypotonic lysis buffer for 10 min at room temperature. Cells were then spun down and stained with antibodies for the Vα2 TCR chain, which is the □ chain, used in the OT-II transgenic TCR. Antibody purchased from BD Pharmingen.

2.2.3 Flow Cytometry

Cells were prepared from thymi, spleen, lymph node and bone marrow. Organs were disaggregated by passage through a 70μm cell strainer into 5ml of RPMI media. The strainer was washed with a further 5ml of RPMI media and cells collected into a 50ml falcon tube. For thymi and spleens, the volume was topped up to 50ml before counting. Cells were re-suspended to $20x10^6$/ml in RPMI media and then 100μl was aliquoted into FACs tubes. Antibodies diluted 1:100 in FACs buffer, and then an equal volume was added to the cells and incubated for at least 20 min on ice. Cells were then washed in FACs buffer and spun down. If a secondary antibody was required this was added 1:200 in FACs buffer for a minimum of 20 min on ice. Cells were then washed and re-suspended in 300μl of FACs buffer. For intracellular staining, cells were fixed in 4% (final) parafomaldehyde for 10 min at room temperature and then permeabilised in 1ml of 0.3% Saponin buffer for 10 min at room temperature. Cells were then washed in 0.1% Saponin buffer before antibodies were added 1:100 in 0.1% Saponin buffer for an hour at room temperature. Cells were washed and any secondary antibodies added 1:200 in 0.1% Saponin for at least 30 min at room temperature, before being washed and then re-suspended in 300μl of FACs buffer. Cells were analysed on either a FACs calibur or LSR II (both Becton Dickinson) using either Cell Quest or FACS diva software. Cells were sorted by either a MoFlo sorter (DakoCytomation) or a FACs aria (Becton Dickinson) to greater than 95% purity. Antibodies against CD4 (RM4-5), CD25 (7D4),

CD90.2 (53-2.1), CD45.1 (A20), CD45.2 (104), CD152 (UC10-4F10-11), TCRβ (H57–59), Vα2 (B20.1), CD45RB FITC (16A), and CD103 (M290) for flow cytometry were purchased from BD Pharmingen. Additionally various streptavidin conjugated reagents were also purchased from BD Pharmingen and Caltag. Antibodies against GITR (BAF524) were purchased from R&D systems; antibodies against CD8α (5H10) and CD45RB tricolour (16A) were purchased from Caltag. Foxp3 staining kit was purchased from ebioscience and staining performed to manufacturer's guidelines.

Intracellular staining for phosphorylated ERK (p-ERK) and Egr-1 was performed as previously described (Costello et al., 2004). Briefly, fixed cells were washed with PBS and permeabilised by addition of 1ml of ice cold methanol for 30 min on ice. Cells were then washed with PBS plus 10% heat inactivated Fetal Calf Serum. Anti-p-ERK (Cell Signalling technologies) or anti-Egr-1 (Santa-Cruz C-19) were added at 1:100 final dilution and incubated on ice for an hour. Cells were washed and the secondary anti-mouse cy-2 (Jackson Laboratories) was added 1:100 for 30 min on ice. Cells were washed and analysed by FACs.

2.2.4 Bone Marrow Reconstitutions

Bone marrow was flushed from femurs of young adult mice. Cells were re-suspended in 1ml freezing media and stored in liquid nitrogen until required. Cells were thawed re-suspended in PBS to the required volume. For mixed bone marrow chimera experiments cells were mixed at a 1:1 ratio. Cells were then passed through a 70μm cell strainer prior to injection. Mice were sub-lethally irradiated with 2 doses of 500 rad given 3 hours apart. 200μl of cells were injected intravenously after 2 hours.

2.2.5 Preparation of APCs

Where experiments required APCs, irradiated, T cell depleted splenocytes were used. Cells were isolated from spleens as previously described and re-suspended to $10x10^6$/ml in FACs buffer. Washed Pan-T cell magnetic beads (Dynal) were added 1:80 and incubated at 4°C on a roller for at least 20 min. T cells were then removed through magnetic separation. Supernatant containing non-T cells was removed and counted.

Cells were then re-suspended in RPMI media and irradiated with 37Gy, then incubated at 37°C until needed.

2.2.6 Cell stimulation

Prior to stimulation cells were rested at 37°C for at least 2 hours. T cells were re-suspended in RPMI media. For short timecourses, cells were stimulated with 10μg/ml soluble αCD3 (2C11) for 20 min on ice. Cells were then washed and re-suspended in serum free media. Cells were warmed to 37°C and then crosslinked with the addition of 75μg/ml goat anti-hamster (Jackson laboratories). Stimulation was terminated by fixation with 4% (final) paraformaldehdye or into lysis buffer for RNA preparation. For longer timecourses cells were spun directly onto plates coated with 10μg/ml αCD3. In some cases irradiated T depleted splenocytes were used as APCs and added at a 1:2 ratio of T cell:APC along with 20ng/ml IL-2 (Chiron).

2.2.7 Immunoblot analysis

For p-ERK detection - Cells were stimulated as described above and the activation reaction stopped by addition of western reducing sample buffer. Samples were run on a 4-12% gradient denaturing gels (Invitrogen) alongside SeeBlue Plus2 markers (Invitrogen), at 120 volts for 1.5 hours in MOPS buffer (Invitrogen) according to manufacturer's guidelines. Gels were then transferred by semi-wet transfer at 200mA for 1hr. Membrane was then blocked for an hour in blocking buffer at room temperature. The membrane was then washed 3x 5 min in wash buffer, before primary antibody was added in western antibody buffer and incubated overnight at 4°C. Membranes were washed as before and then a HRP conjugated antibody was added (DakoCytomation) in western antibody buffer and incubated at room temperature for 1.5 hours. Membranes were washed as before with a final wash in PBS. Excess PBS was drained off before adding ECL (GE Healthcare) to the membrane. Film was then exposed to ECL and developed. If it was necessary to perform a reprobe, the membrane was stripped by washing 2x 10 min in stripping buffer.

Foxp3 detection was performed on 10% polyacrylamide SDS gels and run at 100v for 1.5hours. Gels then analysed as above.

Antibody dilutions

mouse anti-p-ERK (Sigma)	1:1000
mouse anti-pan-ERK (Sigma)	1:1000
rabbit polyclonal anti-Foxp3	1:1500
anti-mouse HRP	1:1500
anti-rabbit HRP	1:1000

10% SDS gel	10ml Total 4.8ml H_20 2.5ml Acrylamide 2.5ml Tris HCL 1.5M pH8.8 100µl 10% SDS 100µl 10% APS 4µl TEMED
Stacking Gel	4ml Total 2.9ml H_20 0.5ml Acrylamide 0.5ml Tris HCl 1M pH6.8 40µl 10% SDS 40µl 10% APS 4µl TEMED

2.2.8 *In vitro* suppression assay

Cells were isolated from thymus or lymph node and $CD4^+CD25^-$ and $CD4^+CD25^+$ populations sorted to greater than 95% purity. Cells were counted and re-suspended to $0.5x10^6$/ml in RPMI media. For thymidine incorporation assays $2.5x10^4$ effector cells ($CD4^+CD25^-$) were plated out onto 96 round bottom plates and co-cultured with $5.0x10^4$ APCs, 2µg/ml soluble αCD3 (2C11) and various ratios of $CD4^+CD25^+$ T cells for 72 hours at 37°C. Cultures were pulsed with 1µCi/well [^{3}H] thymidine for the last 6 hours. After 72 hours incubation, cells were harvested and analysed. For CFSE labelled assays, $CD4^+CD25^-$ T cells were isolated from SJL mice

so they could be identified with the CD45.1 marker. They were re-suspended to $10x10^6$/ml in serum free RPMI media and labelled with 5μM CFSE for 5 min at 37°C. Cells were then washed 3x in RPMI media before being re-suspended to $0.5x10^6$/ml. These cells were then co-cultured as described above with the APCs and T_{regs} ($CD4^+CD25^+$) derived from BL6 mice (CD45.2). After 72 hours cells were analysed by FACs. For suppression assays with cells isolated from thymi, the cells were depleted of $CD8^+$ T cells prior to sorting using CD8 magnetic beads (Dynal). Briefly - thymi were re-suspended to $20x10^6$/ml in FACs buffer. Washed CD8α magnetic beads were added at a 1:20 dilution and incubated on a roller at 4°C for at least 20 min. Cells then separated through magnetic separation and counted. Cells re-suspended to $20x10^6$/ml and stained with CD8α, CD4 and CD25 as previously detailed and sorted to greater than 95% purity.

2.2.9 Pre-activation Suppression Assay

For pre-activation experiments cells were isolated from lymph nodes and sorted for $CD4^+CD25^+$ T cell population to greater than 95% purity. Cells were re-suspended to $1.5x10^6$/ml and incubated at 37°C for 72 hours in the presence of 0.5μg/ml αCD3, 2μg/ml αCD28, 20ng/ml IL-2 and where stated UO126 at 10μM. Cells were then washed in RPMI media and rested at 37°C for at least 2 hours prior to use. Cells were then re-suspended to $0.5x10^6$/ml and co-cultured with $CD4^+CD25^-$ T cells as described above for a thymidine incorporation *in vitro* suppression assay.

2.2.10 *In vivo* model of Inflammatory Bowel Disease (IBD)

Cells were isolated from spleens, and in the case of the double TCF knockout experiment cells were also isolated from lymph nodes. Prior to sorting cells were enriched for $CD4^+$ T cells. Red blood cells were lysed in hypotonic lysis buffer at room temperature for 5 min (maximum of 3 spleens / 5ml). Cells were topped up with PBS and counted. Cells were then re-suspended to $20x10^6$/ml in FACS buffer where anti-CD8α (53-6.7), anti-Mac-1 (M1/70) and anti-B220 (RA3-6B2) (purchased from BD pharmingen) were added 1:100 for 30 min on ice. Cells were washed in FACs buffer

and then anti-sheep IgG magnetic beads (Dynal) were added at 1:20 for 30 min at 4°C on a roller. Cells were separated by magnetic separation and the supernatant counted. Cells were re-suspended at $10x10^6$/ml and sorted into $CD4^+CD45RB^{hi}CD25^-$ and $CD4^+CD45RB^{lo}CD25^+$ populations. Cells were passed though a 70μm cell strainer prior to injection. $RAG2^{-/-}$ mice were injected intraperitoneally with $4x10^5$ $CD4^+CD45RB^{hi}CD25^-$ T cells alone or were co-injected with either $2x10^5$ (1 T_{reg}:2 effector ratio) or $1x10^5$ (1 T_{reg}:4 effector ratio) $CD4^+CD45RB^{lo}CD25^+$. Mice were observed daily and weighed weekly. Any mice showing clinical signs of severe disease were sacrificed accordingly. Any mice losing 20% of their weight were sacrificed in accordance with CRUK ethics guidelines.

2.2.10.1 Histology

Tissue sections were stained with H&E as well as alcian blue (Bancroft and Gamble 2002). Colitis severity was graded semi-quantitatively from 0-4.

2.2.11 Peripheral Conversion

Cells were sorted on CD4 and CD25 to greater than 95% purity. Cells were counted and re-suspended to $0.4x10^6$/ml, and then 1ml was plated out onto 24 well plates and incubated with APCs ($1x10^6$/ml), soluble αCD3 (2μg/ml) and IL-2 (20ng/ml) and TGFβ (2ng/ml). Where stated, UO126 (10μM) was also added. Cells were incubated for 72 hours unless otherwise stated and then analysed for Foxp3 expression by FACs.

2.2.12 Gene Expression Analysis

RNA was prepared using RNeasy Mini kit (Qiagen) according to the manufacture's instructions, including the on column DNase digestion step (Qiagen). 2-50ng of RNA was reverse transcribed using superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA was analysed by real-time

PCR on an ABI7000 machine (Applied biosystems) using either TaqMan probes (Applied biosystems) or SYBR green incorporation (Invitrogen). Expression levels were normalised to GAPDH or HPRT. The following primers and probes were used. Foxp3 and HPRT was performed using published primers and probes (Hori et al., 2003). GAPDH primers designed by S. Guettler, Egr-1 primers designed by R. Nicolas.

TaqMan reactions were made up to 25μl. Taq was purchased from Applied biosystems and used according to the manufacture's guidelines. Primers were added at 0.4μM and probe was added at 0.2μM. SYBR green reactions were made up to 20μl and primer concentrations were the same as for TaqMan reactions. SYBR green Taq was purchased from Invitrogen and diluted according to manufacture's guidelines. For each gene analysed, a standard curve was also run generated from the amplicon cloned into the TA vector (Invitrogen) according to the manufacturer's instructions, and then serially diluted.

Gene	5' Primer	3' Primer
SAP-1	5'-ACAACGCCTGCCAAAAAGC	5'-GAAAGACTAGGGCTCGTTGC
Elk1	5'-TCACGGGATGGTGGTGAGT	5'-GTTCTTGCGCAGTCCCCAT
Net	5'-GATGGCGAGTTCAAGCTCCT	5'-TGGTCTTGTTCTTGCGGAGGC
Foxp3	5'-CCCAGGAAAGACAGCAACCTT	5'-TTCTCACAACCAGGCCACTTG
HPRT	5'-TGAAGAGCTACTGTAATGATCAGTCAAC	5'-AGCAAGCTTGCAACCTTAACCA
GAPDH	5'-ACAACTTTTGGCATTGTGGAAG	5'-ACAGTCTTCTGGGTGGCAG
Egr-1	5'-ATTGATGTCTCCGCTGCAGATC	5'-TCAGCAGCATCATCTCCTCCA

Gene	TaqMan Probe
SAP-1	5'-FAM-ATCGAGCCTGTCGCTGCTGCCT
Elk1	5'-FAM-CAAGTTGGTGGATGCAGAGGAGGTGG
Net	5'-FAM-AAGGCAGAAGAAGTGGCCAAGCTGTG
Foxp3	5'-FAM-ATCCTACCCACTGCTGGCAAATGGAGTC
HPRT	5-VIC-TGCTTTCCCTGGTTAAGCAGTACAGCCC
GAPDH	5'-VIC-CTCATGACCACAGTCCATGCCAT

All reactions were run on the following generic program.

Step	Temperature	Time
1	95°C	10 min
2	95°C	30 secs
3	60°C	1 min
4	72°C	1 min
5	72°C	10 min
40 Cycles		

2.2.13 Bandshifts

2.2.13.1 Bandshift cell extracts

Cells were sorted and a minimum of $5x10^5$ cells was required per sample. Cells were re-suspended at $1x10^6$/ml in warmed serum free RPMI media, and placed in a 37°C water bath. Where indicated PDBu (50ng/ml) was added and incubated at 37°C for 10min. Reactions were stopped by placing directly into ice for at least 5 min. Cells were spun down at 3400rpm for 3 min at 4°C, then washed with ice cold PBS and spun down again as before. Cells were then re-suspended in 10µl of Bandshift extract buffer and spun down for 30 min at 13000rpm at 4°C. Supernatant was then removed and stored at -80°C until needed.

2.2.13.2 Bandshift reactions

1µl of cell extracts were mixed with 1µl recombinant SRF DNA binding domain (SRF[133-265]) (produced and purified by the Treisman lab), 1ng probe in 10µl bandshift buffer with the volume being made up to 20µl with dH_2O. Reactions were incubated at room temperature for 30min. For reactions where the interaction with endogenous SRF was examined, the recombinant SRF DNA binding domain was

replaced with Bandshift core SRF dilution buffer. Where indicated 1µl of anti-SAP-1 (SC-13030X, Santa cruz) was added and incubated for a further 30min at room temperature. Total incubation time was always at least one hour. Samples were briefly spun down and loaded onto 5% non-denaturing gels which had been pre-run for 4 hours at 150V. 50ml gels were poured between two glass plates (19cm x20cm) that had previously been siliconised with Dimethyldichlorosilicone solution. Gels were run at 150V for 10 min and then 170V for 3.25 hours. Spare lanes were loaded with bandshift buffer plus extra xylene cyanol. Gels were then dried for 1.5 hours before autoradiography at -80°C.

5% Acrylamide gel	15ml Acrylamide 6ml 10X TBE 100ml H_20 120µl TEMED 480µl 10% APS

2.2.13.3 Bandshift Probes

Probes for bandshift were generated with primers designed to span the first serum response element (SRE) of the c-fos promoter. Probe was generated by PCR from the following primers – 5' CGCACTGCACCCTCGGTGTTGGCTGC and 5' ATGGCTCCCCCCAGGGCTACAGGGAAAG. 14pmol of primers were incubated with c-fos template, dNTP, Taq (bioline) and p32 CTP. PCR was performed on the following programme:

Step	Temperature	Time
1	95°C	2 min
2	95°C	1 min
3	60°C	1 min
4	72°C	1 min
5	72°C	5 min
30 Cycles		

PCR product was run out on 5% non-denaturing gels which had been pre-run at 170V for 1 hour. Gels were run at 170V for approx 1.5 hours until the Xylene cyanol dye reached half way. The band was then excised and 450µl of extraction buffer was added. This was incubated at 37°C overnight. The following morning this was split into two tubes and an additional 150µl of extraction buffer was added and incubated for a further hour at 37°C. This was then spun down, placed into two fresh tubes and 2.5 volume of ethanol was added. The probe precipitated by placing at -20°C for at least an hour. Probe was then spun down for 10min at 13000rpm in a microcentifuge. The supernatant was then removed and the probe washed with 70% ethanol and was allowed to air dry. The tubes were then re-suspended in 100µl of TEN.

2.2.14 Statistical analysis

Where indicated analysis performed using unpaired T-test except for colitis scores which were analysed using Mann Whitney Test. P less than 0.05 were considered to be significant. Analysis performed using PRISM software.

3 Results – SAP-1 and Regulatory T cell development

3.1 Abstract

The Ternary Complex Factors (TCFs) are a family of Ets domain transcription factors consisting of SAP-1, Elk-1 and Net that interact with the serum response factor (SRF) to regulate the expression of immediate early genes such as Egr-1. As targets of ERK signalling, which has been shown to be important in many immune process, it was conceivable that the TCFs would also be important in the immune system. Indeed deletion of SAP-1 resulted in a substantial defect in positive selection (Costello et al., 2004). Regulatory T cells were initially thought to be positively selected in a similar manner to 'conventional' $CD4^+$ SP thymocytes. In this chapter I address regulatory T cell development in SAP-$1^{-/-}$ animals. If regulatory T cells were selected in a manner similar to 'conventional' T $CD4^+$ SP thymocytes then it would be predicted that there would be a defect in T_{reg} development in SAP-$1^{-/-}$ mice. In contrast the data presented here demonstrates that T_{reg} development is independent of the TCF SAP-1.

3.2 Introduction

The ternary complex factors (TCFs) are a family of Ets domain transcription factors, comprising the SAP-1, Elk-1 and Net proteins. The TCFs are targets of ERK signalling and upon activation regulate a specific set of immediate early genes through interactions with their transcription factor partner serum response factor (SRF). Since TCFs are targets of ERK signalling and regulators of immediate early genes such as Egr-1, previous studies in the transcription laboratory set out to study their developmental role using mouse knockout models. SAP-$1^{-/-}$ mice display a defect in thymocyte positive selection with approximately a 50% reduction in the numbers of $CD4^+$ and $CD8^+$ SP thymocytes (Costello et al., 2004). Furthermore this defect was reproduced in bone marrow reconstitution experiments. Earlier stages of thymocyte

development were also examined, including β-selection. Intriguingly despite a reported role for ERK at this stage of thymocyte development, no defect in SAP-$1^{-/-}$ animals was observed.

Negative selection is the process by which thymocytes that have the potential to be activated by self-peptide in the periphery are deleted from the immune repertoire. It is thought that high affinity interactions between the TCR and self-peptide MHC in the thymus trigger this deletion. In contrast to thymocyte positive selection, negative selection is not affected by the loss of SAP-1 when examined using the HY transgenic TCR model.

Negative selection is not the only mechanism employed by the immune system to guard against autoimmunity. An important class of T cells known as regulatory T cells (T_{regs}) play an essential role in the maintenance of immune tolerance. Regulatory T cells are in part thymically derived and an understanding of their development is thus an important question in immunology. Given the impact of the SAP-1 deletion on the development of 'conventional' SP T cells I decided to examine the role of SAP-1 in regulatory T cell development.

3.3 $CD4^+CD25^+$ T cell development in SAP-$1^{-/-}$ mice

Initially thymically derived T_{regs} were identified as $CD4^+CD25^+$ T cells through the work of Sakaguchi and others (reviewed in Sakaguchi, 2004; and Shevach, 2000). Whilst the expression of CD25 is not specific for regulatory T cells (it is also up-regulated on activated T cells), the majority of the T cells that exhibit suppressive activities could be detected within the $CD4^+CD25^+$ population (Read et al., 2000; Sakaguchi et al., 1995; Piccirillo et al., 2002; Takahashi et al., 1998; Thornton and Shevach, 1998). The expression of CD25 on $CD4^+$ single positive (SP) thymocytes in SAP-$1^{-/-}$ animals was examined. As previously reported, when CD4 and CD8 expression is analysed in SAP-$1^{-/-}$ thymocytes a 50% reduction in the proportion of $CD4^+$ SP thymocytes is observed (Figure 3.1A top panels and Costello et al., 2004). Whilst a clear reduction in $CD8^+$ SP thymocytes was not observed at this level of analysis, gating on thymocytes TCR^{hi} (or HSA^{low}) to exclude immature single positive thymocytes demonstrated a 50% reduction in the proportion of $CD8^+$ SP thymocytes (Costello et al., 2004). Thus SAP-1 contributes to both $CD4^+$ and $CD8^+$ positive

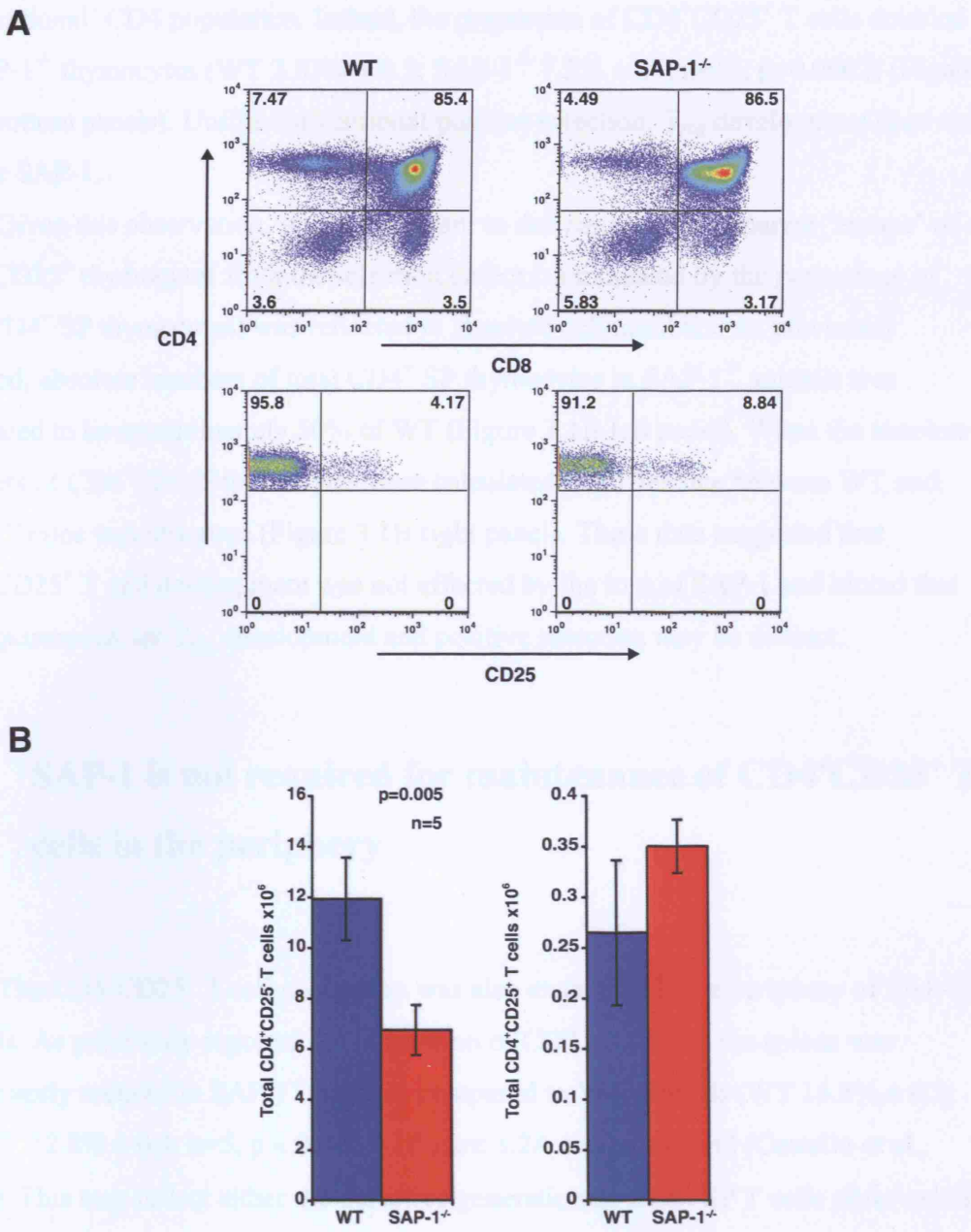


Figure 3.1. Normal numbers of $CD4^+CD25^+$ T cells develop in SAP-$1^{-/-}$ animals. A. Top panels, CD4 and CD8 expression on developing thymocytes. Bottom panels, proportion of $CD25^+$ T cells on $CD4^+$ gated T cells. **B.** Numbers of $CD4^+CD25^-$ (left) and $CD4^+CD25^+$ thymocytes (right) in WT and SAP-$1^{-/-}$ animals (n=5).

selection. Surprisingly examination of the $CD4^+CD25^+$ component of the $CD4^+$ population did not reveal a 50% reduction compared to WT, as had been seen in the 'conventional' CD4 population. Indeed, the proportion of $CD4^+CD25^+$ T cells doubled in SAP-$1^{-/-}$ thymocytes (WT 3.83% ± 0.3; SAP-$1^{-/-}$ 7.2% ±0.7; n=15, p=0.0002) (Figure 3.1A bottom panels). Unlike conventional positive selection, T_{reg} development does not require SAP-1.

Given this observation, it was important to determine if the apparent 'escape' of $CD4^+CD25^+$ thymocytes from the selection defect (as indicated by the percentage of total $CD4^+$ SP thymocytes) was reflected in absolute cell numbers. As previously reported, absolute numbers of total $CD4^+$ SP thymocytes in SAP-$1^{-/-}$ animals was calculated to be approximately 50% of WT (Figure 3.1B left panel). When the absolute numbers of $CD4^+CD25^+$ thymocytes were calculated no difference between WT and SAP-$1^{-/-}$ mice was observed (Figure 3.1B right panel). These data suggested that $CD4^+CD25^+$ T cell development was not affected by the loss of SAP-1 and hinted that the requirements for T_{reg} development and positive selection may be distinct.

3.4 SAP-1 is not required for maintenance of $CD4^+CD25^+$ T cells in the periphery

The $CD4^+CD25^+$ T cell population was also examined in the periphery of SAP-$1^{-/-}$ animals. As previously reported the proportion of $CD4^+$ T cells in the spleen was significantly reduced in SAP-$1^{-/-}$ animals compared to WT controls (WT 16.8% ± 0.2; SAP-$1^{-/-}$ 12.8% ± 0.4; n=5, $p < 0.0001$) (Figure 3.2A top panels and (Costello et al., 2004)). This may reflect either the defective generation of $CD4^+$ SP T cells observed in the SAP-$1^{-/-}$ thymus or defects in $CD4^+$ T cell homeostasis. Interestingly analysis of the proportion of $CD4^+CD25^+$ T cells revealed this population to be substantially increased in SAP-$1^{-/-}$ animals compared to WT controls (WT 13.2% ± 0.2; SAP-$1^{-/-}$ 20.2% ± 1.6; n=5, p=0.002) (Figure 3.2A bottom panels). Once again, when the numbers of $CD4^+CD25^+$ T cells was calculated no change in the absolute numbers was observed (Figure 3.2B right panel). This observation was reminiscent of what was seen in the thymus. Furthermore when the total numbers of $CD4^+$ SP T cells was calculated, a significant reduction was observed although this is a smaller reduction than the 50%

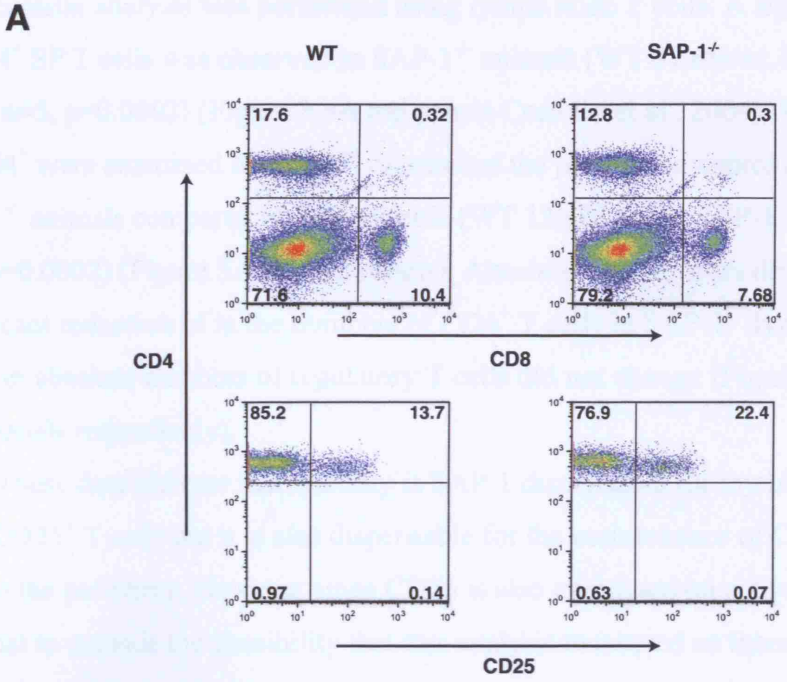


Figure 3.2. Normal numbers of CD4$^+$CD25$^+$ T cells are present in spleens of SAP-1$^{-/-}$ spleens. A. Top panels, CD4 and CD8 expression on splenocytes. Bottom panels, proportion of CD25$^+$ T cells on CD4$^+$ gated T cells. **B.** Numbers of CD4$^+$CD25$^-$ (left) and CD4$^+$CD25$^+$ splenocytes (right) in WT and SAP-1$^{-/-}$ animals (n=5).

reduction observed in the thymus, possibly suggestive of some lymphopenic compensation (Figure 3.2B left panel and Costello et al., 2004).

Similar analysis was performed using lymph node T cells. A significant reduction in $CD4^+$ SP T cells was observed in SAP-$1^{-/-}$ animals (WT 31.6% ±1.8; SAP-$1^{-/-}$ 19.0% ± 0.8; n=5, p=0.0002) (Figure 3.3A top panels Costello et al., 2004). When cells gated on $CD4^+$ were examined for $CD25^+$ expression the proportion approximately doubled in SAP-$1^{-/-}$ animals compared to WT controls (WT 12.4% ± 0.6; SAP-$1^{-/-}$ 27.7% ± 2.3; n=5, p=0.0002) (Figure 3A bottom panels). Absolute cell numbers demonstrated a significant reduction of in the numbers of $CD4^+$ T cells in SAP-$1^{-/-}$ lymph nodes however absolute numbers of regulatory T cells did not change (Figure 3.3B left and right panels respectively).

These data indicate that not only is SAP-1 dispensable for thymic generation of $CD4^+CD25^+$ T cells but it is also dispensable for the maintenance of $CD4^+CD25^+$ T cells in the periphery. However since CD25 is also expressed on activated T cells, it was essential to exclude the possibility that this analysis measured an increase in effector $CD4^+$ T cells displaying an activated phenotype even though the mice were housed under SPF conditions.

3.5 The $CD4^+CD25^+$ thymocytes that escape the SAP-1 positive selection defect express regulatory T cell markers

Along with CD25, regulatory T cells are known to express high levels of a number of cell surface markers that correlate with suppressor activity. These include CTLA-4 and the TNFR family member GITR. Additionally CD103 has been found to be expressed on around 20% of regulatory T cells. When SAP-$1^{-/-}$ $CD4^+CD25^+$ thymocytes were examined for these markers it was found that they expressed high levels of GITR and CTLA-4 at equivalent levels to WT $CD4^+CD25^+$ thymocytes (Figure 3.4A top and middle panels). Likewise these cells also expressed CD103 at equivalent levels to WT $CD4^+CD25^+$ T cells (Figure 3.4A bottom panel). Therefore the $CD4^+CD25^+$ thymocytes present in SAP-$1^{-/-}$ animals are phenotypically equivalent to WT $CD4^+CD25^+$ thymocytes and likely to be regulatory in their nature.

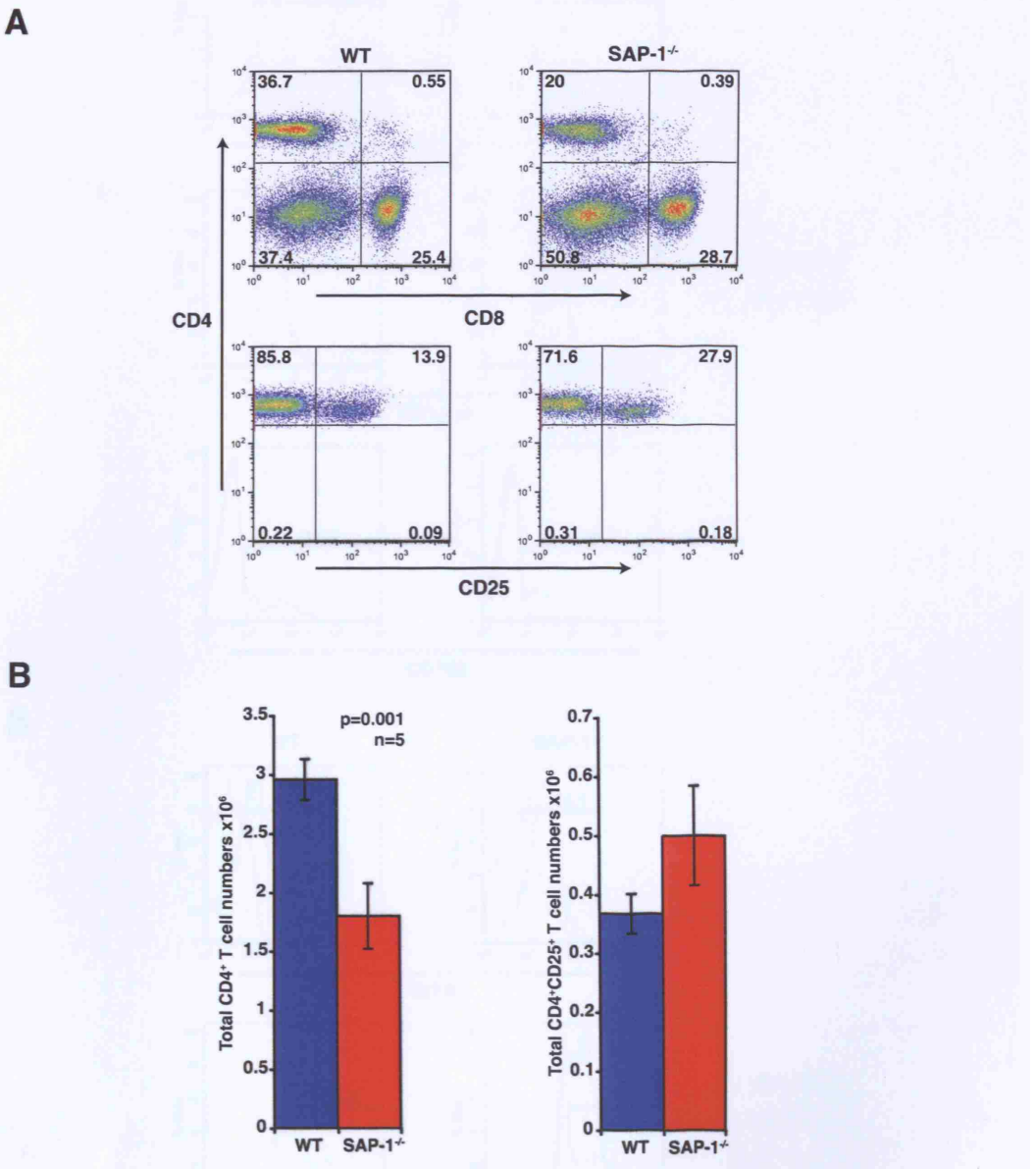
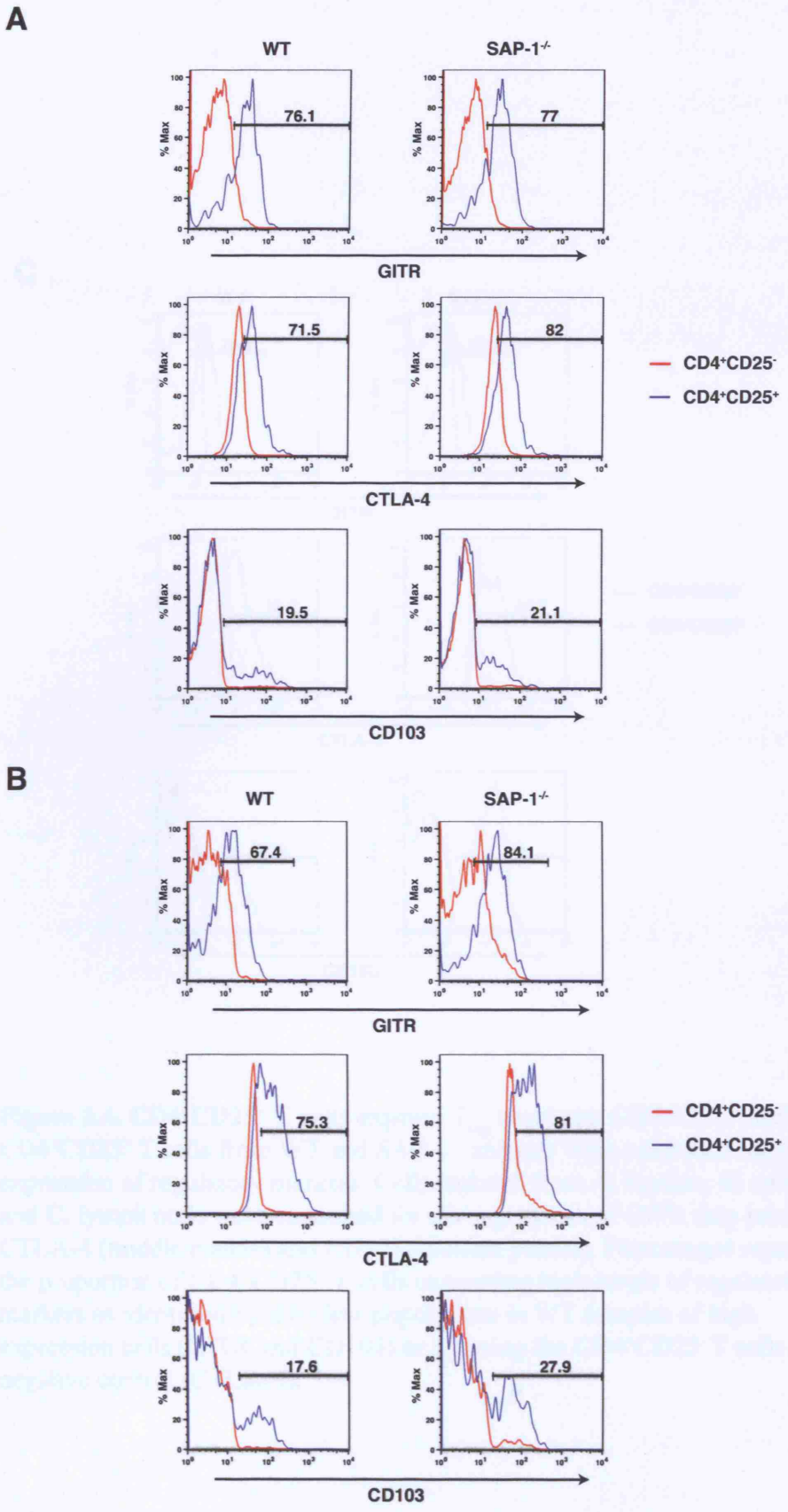


Figure 3.3. Normal numbers of $CD4^+CD25^+$ T cells are present in SAP-$1^{-/-}$ lymph nodes. A. Top panels, CD4 and CD8 expression on lymph node cells Bottom panels, proportion of $CD25^+$ T cells on $CD4^+$ gated T cells.
B. Numbers of $CD4^+CD25^-$ (left) and $CD4^+CD25^+$ thymocytes (right) in WT and SAP-$1^{-/-}$ animals (n=5).


A
WT
SAP-1-/-
76.1
77
% Max
GITR
71.5
82
CTLA-4
19.5
21.1
CD103
CD4+CD25-
CD4+CD25+
B
WT
SAP-1-/-
67.4
84.1
GITR
75.3
81
CTLA-4
17.6
27.9
CD103
CD4+CD25-
CD4+CD25+

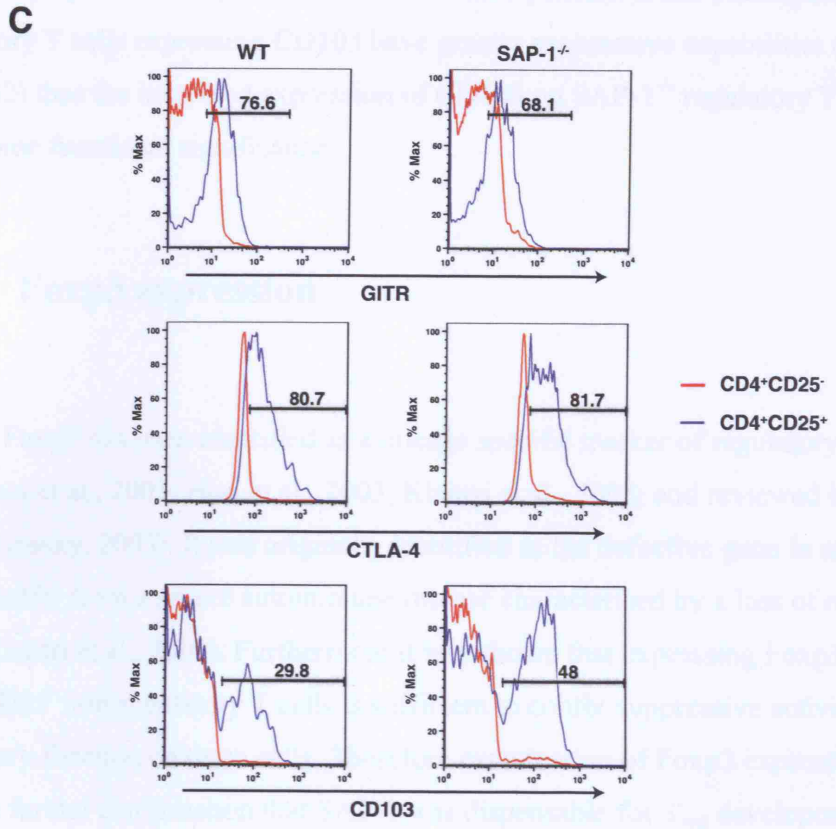


Figure 3.4. $CD4^+CD25^+$ T cells express T_{reg} markers. $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from WT and SAP-$1^{-/-}$ animals were examined for the expression of regulatory markers. Cells isolated from **A.** thymus, **B.** spleen and **C.** lymph node were examined for the expression of GITR (top panels), CTLA-4 (middle panels) and CD103 (Bottom panels). Percentages represent the proportion of $CD4^+CD25^+$ T cells expressing high levels of regulatory markers as identified by the clear populations in WT samples of high expression cells (GITR and CD103) or by using the $CD4^+CD25^-$ T cells as a negative control (CTLA-4).

The expression of these markers was also assessed on peripheral $CD4^+CD25^+$ T cells from SAP-$1^{-/-}$ mice (Figure 3.4B and C). GITR and CTLA-4 were expressed on SAP-$1^{-/-}$ $CD4^+CD25^+$ T cells at equivalent levels to that seen in cells from WT animals (Figure 3.4B and C top and middle panels). Intriguingly a greater proportion of SAP-$1^{-/-}$ $CD4^+CD25^+$ T cells expressed CD103, increasing from 15.7% ± 1.0 to 29.6% ± 1.0 (n=3, p=0.0007) in SAP-$1^{-/-}$ spleens and 28.5% ± 1.7 to 48.5% ± 0.2 (n=3, p=0.0003) in SAP-$1^{-/-}$ lymph nodes (Figure 3.4B and C bottom panels). It has been speculated that regulatory T cells expressing CD103 have greater suppressive capabilities (Lehmann et al., 2002) thus the increased expression of CD103 on SAP-$1^{-/-}$ regulatory T cells may have some functional significance.

3.6 Foxp3 expression

Foxp3 has been identified as a lineage specific marker of regulatory T cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; and reviewed in Fontenot and Rudensky, 2005). It was originally identified as the defective gene in scurfy mice which suffer from a severe autoimmune disease characterised by a loss of regulatory T cells (Khattri et al., 2003). Furthermore it was shown that expressing Foxp3 in $CD4^+CD25^-$ non-regulatory T cells is sufficient to confer suppressive activity and regulatory function on these cells. Therefore examination of Foxp3 expression would provide further confirmation that SAP-1 was dispensable for T_{reg} development in the thymus.

Thymocytes were sorted as $CD4^+CD25^-$ or $CD4^+CD25^+$ from both WT and SAP-$1^{-/-}$ animals. mRNA was prepared from these cells and real-time RT-PCR was performed to quantify levels of Foxp3 mRNA. It has previously been shown that Foxp3 mRNA could only be detected in regulatory T cells and not $CD4^+CD25^-$ T cells or activated $CD4^+CD25^+$ T cells (Fontenot et al., 2003; Khattri et al., 2003). Consistent with these observations, Foxp3 mRNA was only detectable in $CD4^+CD25^+$ thymocytes (Figure 3.5A). $CD4^+CD25^+$ thymocytes derived from SAP-$1^{-/-}$ animals displayed similar levels of Foxp3 mRNA to WT $CD4^+CD25^+$ thymocytes suggesting that the SAP-$1^{-/-}$ $CD4^+CD25^+$ thymocytes were indeed regulatory T cells as defined by Foxp3 expression. Similar results were obtained when $CD4^+CD25^+$ T cells were examined from WT and SAP-$1^{-/-}$ lymph nodes (Figure 3.5B).

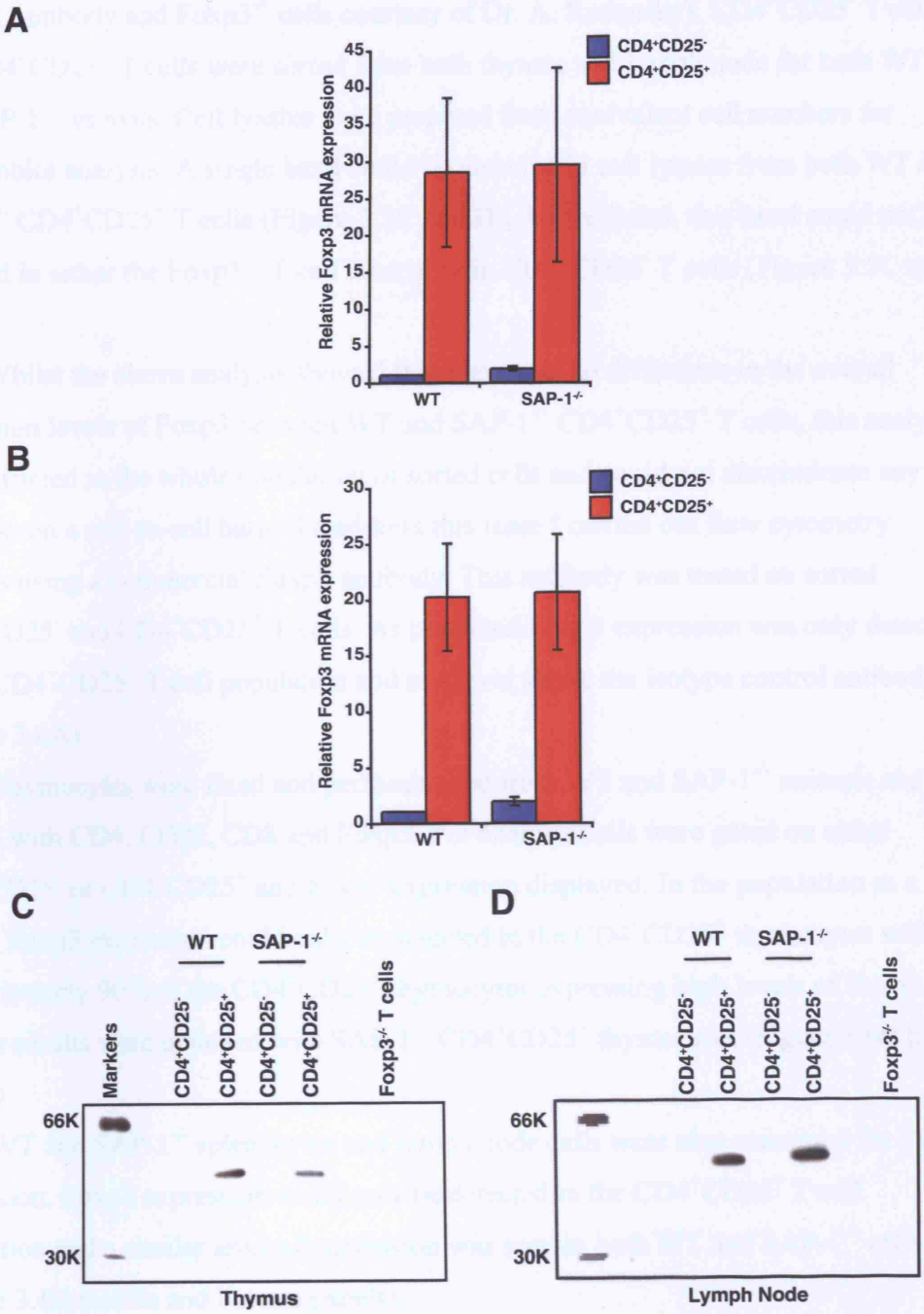


Figure 3.5. The CD4$^+$CD25$^+$ T cell population contains Foxp3$^+$ cells. A. and B. Cells were sorted into CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cell populations from WT and SAP-1$^{-/-}$ animals and examined for Foxp3 mRNA expression by real-time RT-PCR normalised to GAPDH. Three separate RNA preperations were examined in this way. Relative expression in WT CD4$^+$CD25$^-$ T cells given arbitrary value of 1. **A.** Cells prepared from thymus. **B.** Cells prepared from lymph nodes. **C. and D.** Populations sorted as in **A.** and **B.** Total cell extracts then examined for Foxp3 protein expression by immunoblot analysis and run alongside Foxp3$^{-/-}$ T cells. **C.** Cells prepared from thymus. **D.** Cells prepared from lymph nodes.

To examine Foxp3 protein levels, I used antibody specific immunoblotting of Foxp3 (Antibody and Foxp3$^{-/-}$ cells courtesy of Dr. A. Rudensky). CD4^{+}CD25^{-} T cells and CD4^{+}CD25^{+} T cells were sorted from both thymus and lymph node for both WT and SAP-1$^{-/-}$ animals. Cell lysates were prepared from equivalent cell numbers for immunoblot analysis. A single band could be detected in cell lysates from both WT and SAP-1$^{-/-}$ CD4^{+}CD25^{+} T cells (Figure 3.5C and D). As predicted, this band could not be detected in either the Foxp3$^{-/-}$ T cell control or in CD4^{+}CD25^{-} T cells (Figure 3.5C and D).

Whilst the above analysis showed that there was no difference in the overall expression levels of Foxp3 between WT and SAP-1$^{-/-}$ CD4^{+}CD25^{+} T cells, this analysis was restricted to the whole population of sorted cells and could not discriminate any variation on a cell-to-cell basis. To address this issue I carried out flow cytometry analysis using a commercial Foxp3 antibody. This antibody was tested on sorted CD4^{+}CD25^{-} and CD4^{+}CD25^{+} T cells. As predicted Foxp3 expression was only detected in the CD4^{+}CD25^{+} T cell population and at a level above the isotype control antibody (Figure 3.6A).

Thymocytes were fixed and permeabilised from WT and SAP-1$^{-/-}$ animals and stained with CD4, CD25, CD8 and Foxp3. For analysis cells were gated on either CD4^{+}CD25^{-} or CD4^{+}CD25^{+} and Foxp3 expression displayed. In the population as a whole, Foxp3 expression could only be detected in the CD4^{+}CD25^{+} thymocytes with approximately 90% of the CD4^{+}CD25^{+} thymocytes expressing high levels of Foxp3. Similar results were obtained with SAP-1$^{-/-}$ CD4^{+}CD25^{+} thymocytes (Figure 3.6B top panels).

WT and SAP-1$^{-/-}$ splenocytes and lymph node cells were also examined for Foxp3 expression. Foxp3 expression could only be detected in the CD4^{+}CD25^{+} T cell population and a similar level of expression was seen in both WT and SAP-1$^{-/-}$ cells (Figure 3.6B middle and bottom panels).

3.6.1 CD4^{+}Foxp3^{+} T cell data correlate with CD4^{+}CD25^{+} T cells

Since the CD4^{+}CD25^{+} T cell population contained the majority of the Foxp3^{+} T cells, I examined whether the CD4^{+}Foxp3^{+} T cell profile correlates with the increased proportion seen in the CD4^{+}CD25^{+} T cell population. Thymocytes were gated on CD4 and then assessed for Foxp3 expression. In agreement with the CD4^{+}CD25^{+} T cell data,

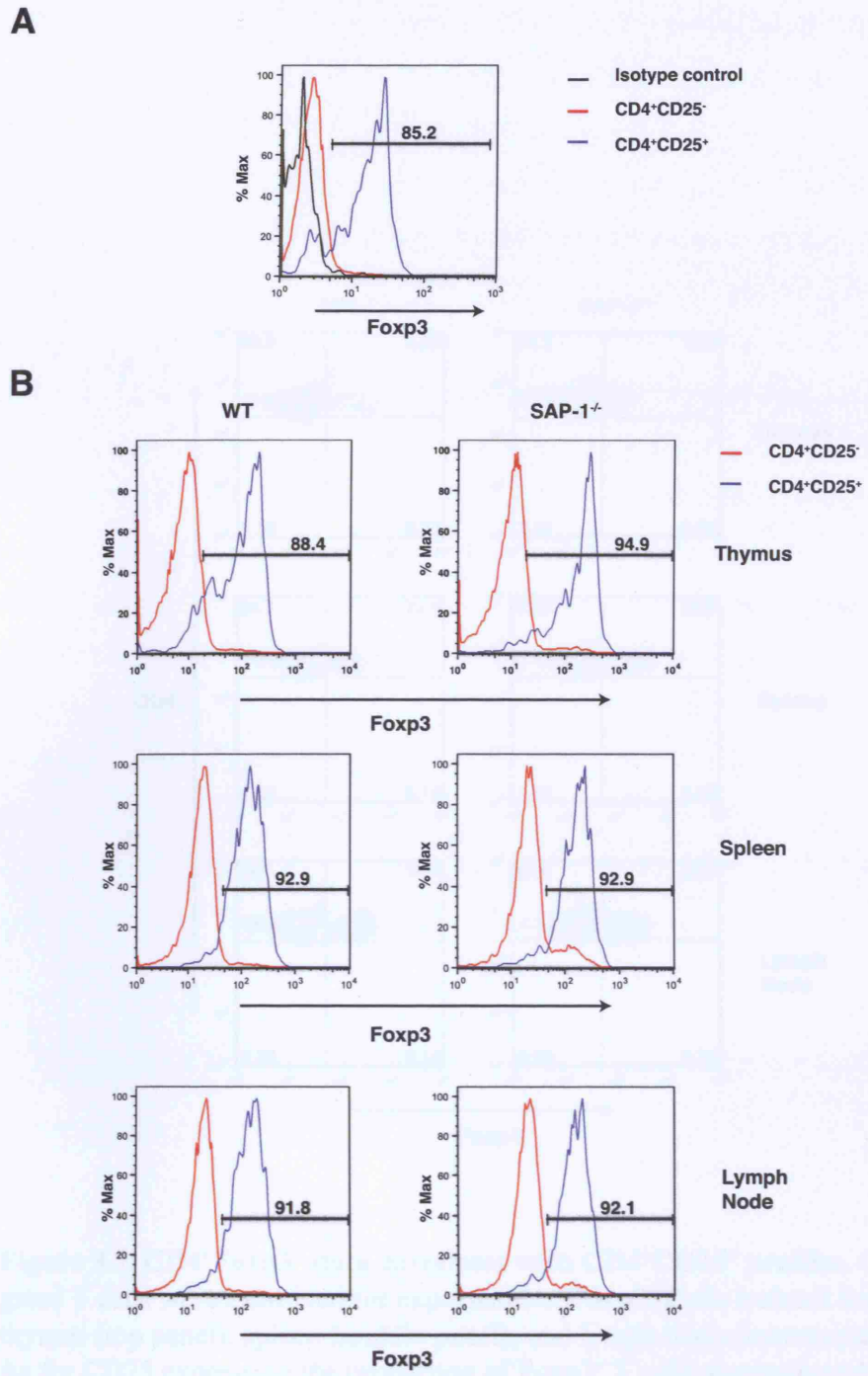


Figure 3.6. CD4$^+$CD25$^+$ T cells express high levels of Foxp3. Foxp3 expression was assessed on a per cell basis by intracellular staining and measured by flow cytometry analysis. **A.** A commercial antibody was used to detect Foxp3 staining. Foxp3 staining could only be detected in CD4$^+$CD25$^+$ T cells. No staining was detected with an isotype control. **B.** The majority of WT and SAP-1$^{-/-}$ CD4$^+$CD25$^+$ T cells expressed high levels of Foxp3. Cells isolated from: thymus (top panels); spleen (middle panels); and lymph node (bottom panels). Percentage represents the proportion of CD4$^+$CD25$^+$ T cells expressing high levels of Foxp3 as determined by using WT CD4$^+$CD25$^-$ T cells as a negative control.

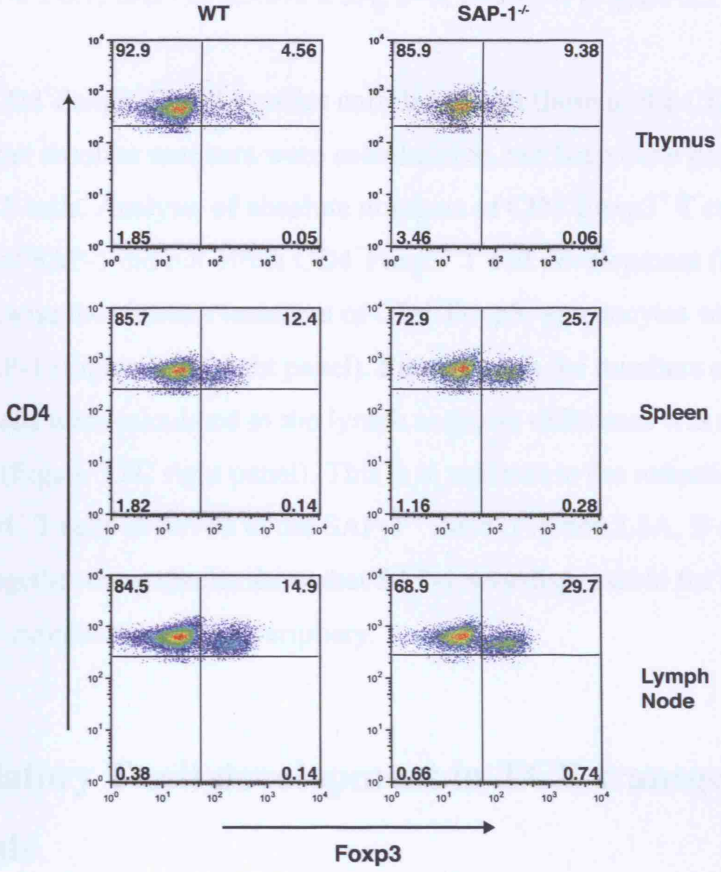


Figure 3.7. CD4$^+$Foxp3$^+$ data correlates with CD4$^+$CD25$^+$ profiles. CD4$^+$ gated T cells were examined for expression of Foxp3. Cells isolated from: thymus (top panel); spleen (middle panel); and lymph node (bottom panel). As for CD25 expression the proportion of Foxp3$^+$ T cells approximately doubled in SAP-1$^{-/-}$ animals.

the proportion of $CD4^+Foxp3^+$ thymocytes approximately doubled in SAP-$1^{-/-}$ animals (WT 4.1% ± 0.1; SAP-$1^{-/-}$ 8.7% ± 0.5; n=4, $p < 0.0001$) (Figure 3.7 top panels). When this analysis was performed on splenocytes, again the $CD4^+Foxp3^+$ T cell population approximately doubled in SAP-$1^{-/-}$ animals (WT 13.1% ± 0.3; SAP-$1^{-/-}$ 23.7% ± 2.5; n=5, p=0.003) (Figure 3.7 middle panels). Cells isolated from lymph nodes were also analysed and again the $CD4^+Foxp3^+$ T cell population was found to approximately double (WT 13.0% ± 0.7; SAP-$1^{-/-}$ 25.4% ± 3.4; n=5, p=0.007) (Figure 3.7 bottom panels).

Since the $CD4^+Foxp3^+$ T cell profiles correlated with those of the $CD4^+CD25^+$ T cell populations the absolute numbers were calculated as had been done previously with the $CD4^+CD25^+$ T cells. Analysis of absolute numbers of $CD4^+Foxp3^+$ T cells showed that inactivation of SAP-1 did not affect $CD4^+Foxp3^+$ T cell development (Figure 3.8A right panel). Likewise the absolute numbers of $CD4^+Foxp3^+$ splenocytes was unaffected by the loss of SAP-1 (Figure 3.8B right panel). Finally when the numbers of $CD4^+Foxp3^+$ T cells were calculated in the lymph node, no difference was observed in SAP-$1^{-/-}$ animals (Figure 3.8C right panel). This is in contrast to the reduction of conventional $CD4^+$ T cells observed in the SAP-$1^{-/-}$ mice (Figures 3.8A, B and C, left panels). Taken together these results show that SAP-1 was dispensable for T_{reg} development and maintenance in the periphery.

3.7 Regulatory T cell development in TCR transgenic animals

The effect of SAP-1 on positive selection appears much more marked when TCR transgenic animals are examined (Costello et al., 2004). Therefore it was asked whether the $CD4^+$ SP thymocytes present in SAP-$1^{-/-}$ OT-II TCR transgenic mice were regulatory T cells. As previously reported a substantial reduction in the proportion of $CD4^+$ SP thymocytes was observed in SAP-$1^{-/-}$ OT-II TCR transgenic mice (Figure 3.9A top panels). This reduction in the proportion of $CD4^+$ SP thymocytes was greater than the 50% observed in polyclonal animals, although the reason for this is not clear (Costello et al., 2004). When the proportion of Foxp3 expressing cells was examined a substantial increase was observed in the SAP-$1^{-/-}$ OT-II TCR transgenic mice (Figure 9A bottom panels). When the numbers of $CD4^+Foxp3^+$ T cells were determined, no

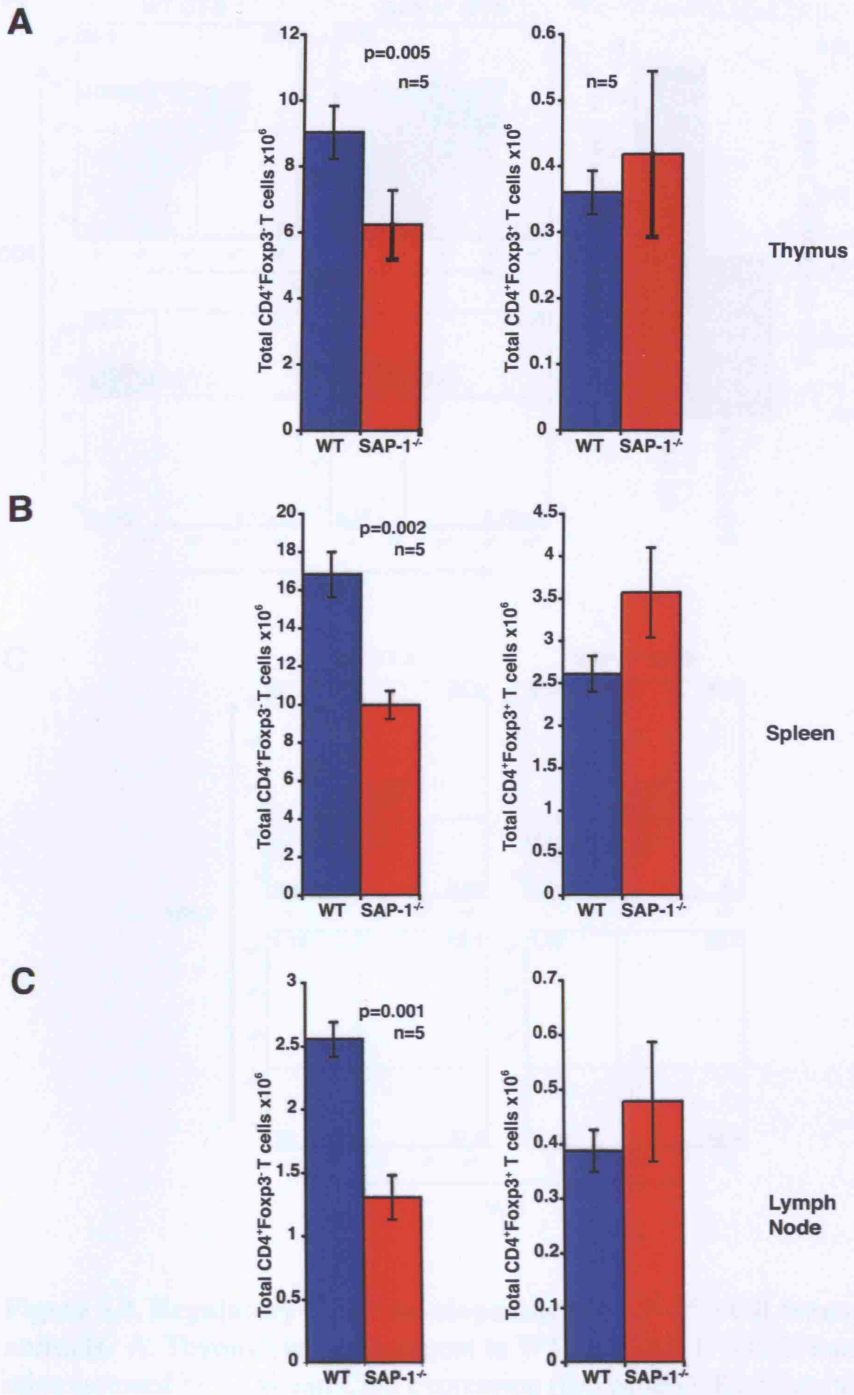


Figure 3.8. Foxp3 numbers are unaffected in SAP-1$^{-/-}$ animals. Absolute numbers were quantified as had previously been done for CD4$^+$CD25$^+$ T cells. Unlike conventional T cells (left panels), no change in the numbers of CD4$^+$Foxp3$^+$ T cells (right panels) was observed in SAP-1$^{-/-}$ animals. Cells isolated from **A.** thymus, **B.** spleen, **C.** lymph node.

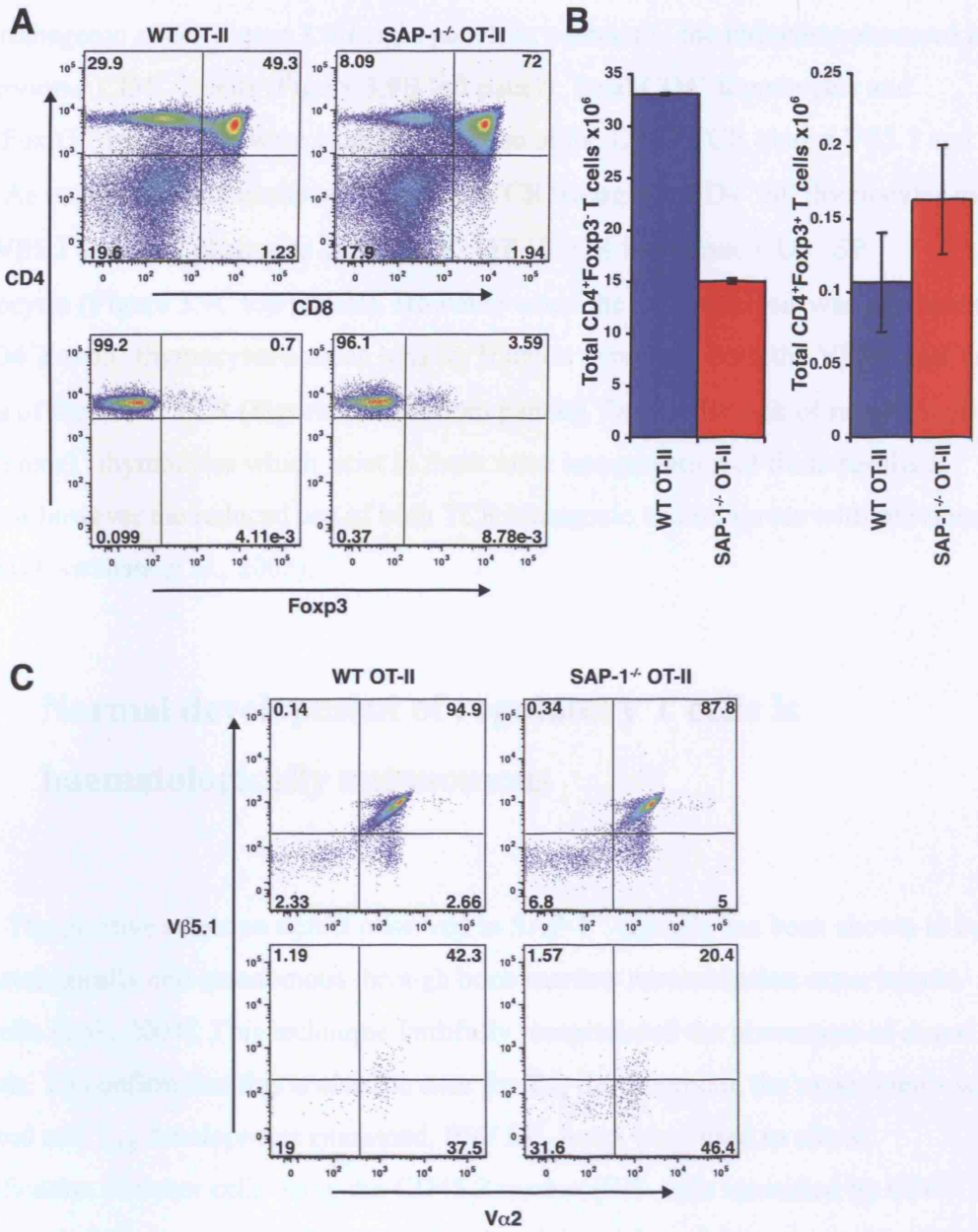
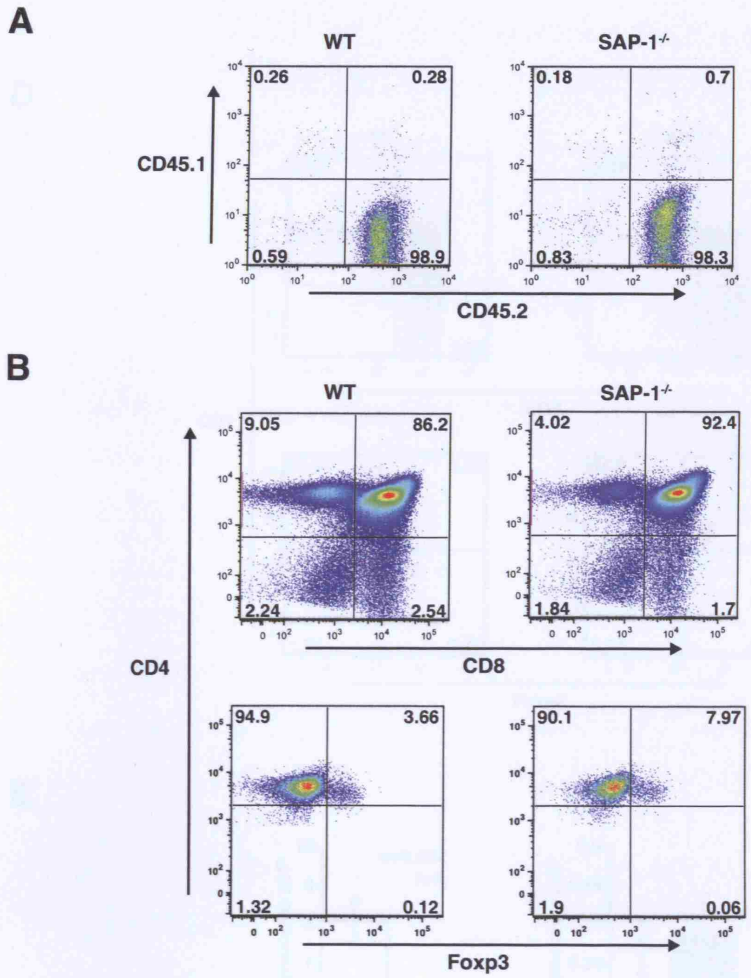


Figure 3.9. Regulatory T cell development in SAP-1$^{-/-}$ TCR transgenic animals. **A.** Thymocyte development in WT and SAP-1$^{-/-}$ OT-II transgenic mice assessed by CD4 and CD8 expression (top panels). Regulatory T cell development assessed by Foxp3 expression (bottom panels). **B.** Total numbers of CD4^{+}Foxp3^{-} thymocytes (left) and CD4^{+}Foxp3^{+} thymocytes (right) in WT and SAP-1$^{-/-}$ OT-II transgenic animals. **C.** Proportions of total CD4^{+} thymocytes (top panels) and CD4^{+}Foxp3^{+} thymocytes (bottom panels) which express the OT-II TCR chains Vβ5.1 and Vα2.

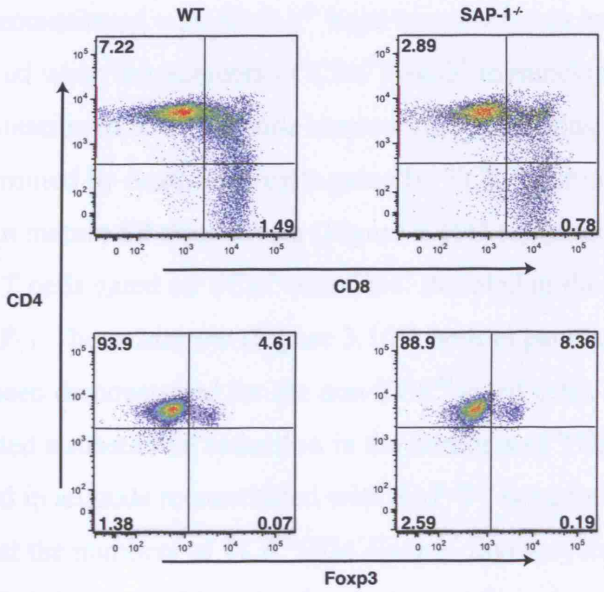
difference was observed between WT OT-II TCR transgenic mice and SAP-1$^{-/-}$ OT-II TCR transgenic mice (Figure 3.9B right panel) in contrast to the reduction observed in conventional CD4$^+$ T cells (Figure 3.9B left panel). Total CD4$^+$ thymocytes and CD4$^+$Foxp3$^+$ thymocytes were examined for use of the OT-II TCR chains Vβ5.1 and Vα2. As expected the majority of WT OT-II TCR transgenic CD4$^+$ SP thymocytes used both Vβ5.1 and Vα2 chains, as did SAP-1$^{-/-}$ OT-II TCR transgenic CD4$^+$ SP thymocytes (Figure 3.9C top panels). However when the same analysis was performed on CD4$^+$Foxp3$^+$ thymocytes a much smaller fraction expressed both the Vβ5.1 and Vα2 chains of the OT-II TCR (Figure 3.9C bottom panels). Due to the lack of numbers of CD4$^+$Foxp3$^+$ thymocytes which exist in these mice interpretation of these results is difficult however the reduced use of both TCR transgenic chains agrees with previous studies (Kawahata et al., 2002).

3.8 Normal development of regulatory T cells is haematologically autonomous

The positive selection defect observed in SAP-1$^{-/-}$ animals has been shown to be haematologically cell autonomous through bone marrow reconstitution experiments (Costello et al., 2004). This technique faithfully recapitulated the phenotype of donor animals. To confirm that this is also the case for T_{reg} development, the experiments were repeated and T_{reg} development examined. Bl6/ SJL hosts were used to allow identification of donor cells using the CD45.2 marker (SJL cells identified by CD45.1 expression). Mice were sub-lethally irradiated and then injected intravenously with bone marrow and allowed to reconstitute for 6-8 weeks. Analysis of CD45.1 and CD45.2 expression showed that greater than 95% of cells in animals reconstituted with either WT or SAP-1$^{-/-}$ bone marrow were derived from the donor bone marrow as judged by expression of the CD45.2 marker (Figure 3.10A). Thus SAP-1$^{-/-}$ bone marrow is capable of efficiently reconstituting lymphopenic mice. Flow cytometry analysis of CD4 and CD8 expression was used to assess thymocyte development of CD45.2$^+$ gated cells. As observed in the donor animals positive selection was severely impaired in animals reconstituted with SAP-1$^{-/-}$ bone marrow with approximately only 4% CD4$^+$ SP thymocytes (Figure 3.10B top panels). When T_{reg} development was assessed by the expression of Foxp3 on CD4$^+$ gated cells the proportion of Foxp3 expressing cells in


A
WT
SAP-1-/-
CD45.1
CD45.2
0.26
0.28
0.59
98.9
0.18
0.7
0.83
98.3
B
WT
SAP-1-/-
CD4
CD8
Foxp3
9.05
86.2
2.24
2.54
4.02
92.4
1.84
1.7
94.9
3.66
1.32
0.12
90.1
7.97
1.9
0.06
C
p=0.001
n=4
Total CD4+Foxp3- thymocytes x10^6
Total CD4+Foxp3+ thymocytes x10^6
WT
SAP-1-/-

D



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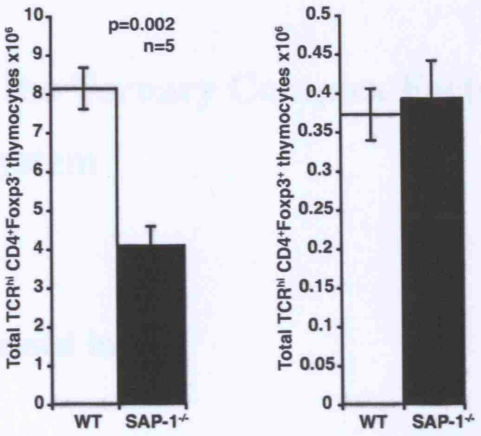


Figure 3.10. SAP-1$^{-/-}$ T_{reg} development is haematalogically autonomous. Mice reconstituted with SAP-1$^{-/-}$ bone marrow were assessed for T_{reg} development. **A.** Reconstitution efficiency was assessed by CD45.2 expression. Further analysis was performed on CD45.2^{+} gated cells. **B.** Foxp3 expression in CD4^{+} gated populations; in mice reconstituted with WT and SAP-1$^{-/-}$ bone marrow. **C.** Numbers of CD4^{+}Foxp3^{-} (left) and CD4^{+}Foxp3^{+} thymocytes (right) in mice reconstituted with WT and SAP-1$^{-/-}$ bone marrow. **D**. CD4 and CD8 expression on TCR^{hi} gated cells (top panels). Regulatory T cell development was assessed by Foxp3 expression in TCR^{hi}CD4^{+} gated populations (bottom panels). **E.** Numbers of TCR^{hi}CD4^{+}Foxp3^{-} thymocytes (left) and TCR^{hi}CD4^{+}Foxp3^{+} thymocytes (right) (n=4).

SAP-1$^{-/-}$ animals had approximately doubled (Figure 10B bottom panels) reproducing what had previously been observed in the donor animals. Furthermore as in the donor animals, a significant reduction in the numbers of CD4^{+} SP thymocytes could be observed in animals reconstituted with SAP-1$^{-/-}$ bone marrow but as expected no difference was observed when the numbers of CD4^{+}Foxp3^{+} thymocytes were calculated (Figure 3.10C). Examination of SAP-1$^{-/-}$ bone marrow reconstitutions for mature T cell development, as determined by examining cells gated by TCR^{hi}, demonstrated a significant reduction in mature SP thymocytes (Figure 3.10D top panel). The proportion of Foxp3^{+} expressing T cells gated on TCR^{hi} and CD4^{+} doubled in the animals reconstituted with SAP-1$^{-/-}$ bone marrow (Figure 3.10D bottom panel), agreeing with what had previously been demonstrated for the non-TCR^{hi} gated cells. Thus when the numbers were calculated a substantial reduction in the numbers of TCR^{hi}CD4^{+} SP T cells was still observed in animals reconstituted with SAP-1$^{-/-}$ bone marrow (Figure 3.10E left panel) whilst the numbers of TCR^{hi}CD4^{+}Foxp3^{+} thymocytes remained constant (Figure 3.10E right panel). Thus the development of regulatory T cells is faithfully recapitulated in WT hosts in bone marrow reconstitution experiments.

3.9 Expression of the Ternary Complex Factors in cells of the Immune system

3.9.1 TCFs are expressed in T_{regs}

Presented with data demonstrating that regulatory T cell development appeared to be unaffected by the loss of SAP-1 it was important to address the following question: was SAP-1 activity simply not required for this developmental process or could it be that other TCF family members could substitute for the loss of SAP-1 activity. As described above the development of effector T cells was severely affected in SAP-1$^{-/-}$ animals. Previously it had been shown that SAP-1 is the predominant TCF in the thymus and this could provide one explanation as to why an immune phenotype is observed in SAP-1$^{-/-}$ animals but not in Elk-1$^{-/-}$ or Net$^{\delta/\delta}$ animals (Cesari et al., 2004; Ayadi et al., 2001). Following this rationale, one hypothesis might be that the lack of effect on T_{reg} development was an indication that SAP-1 is not the predominant TCF in

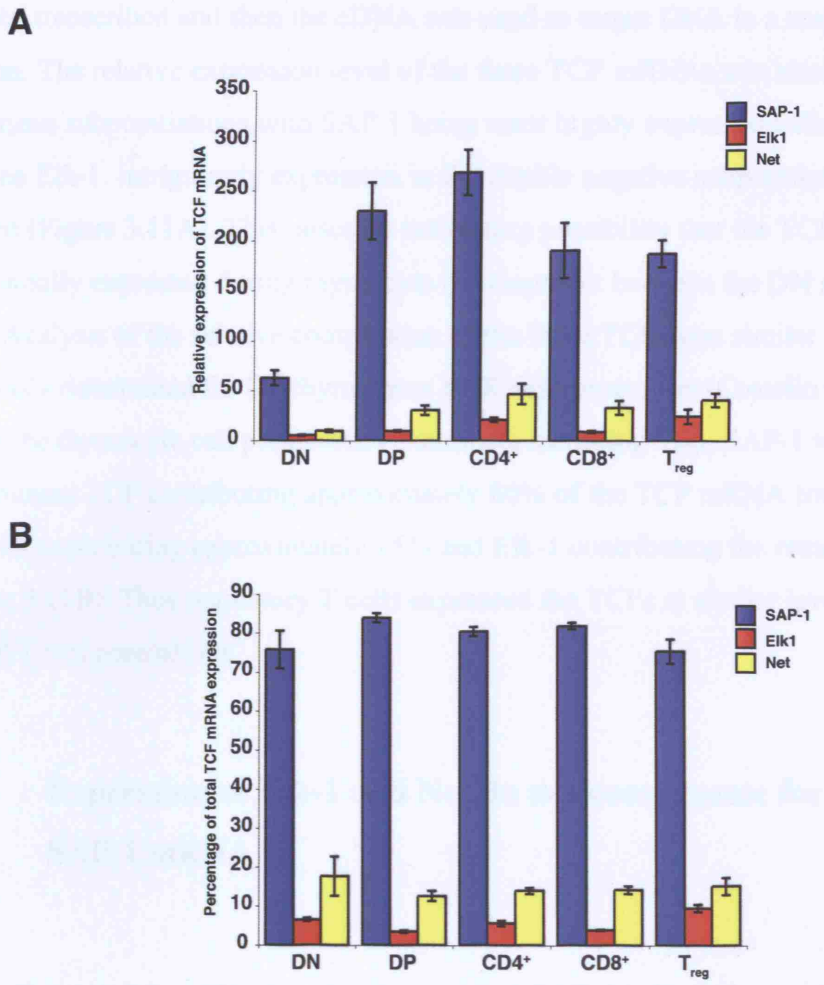


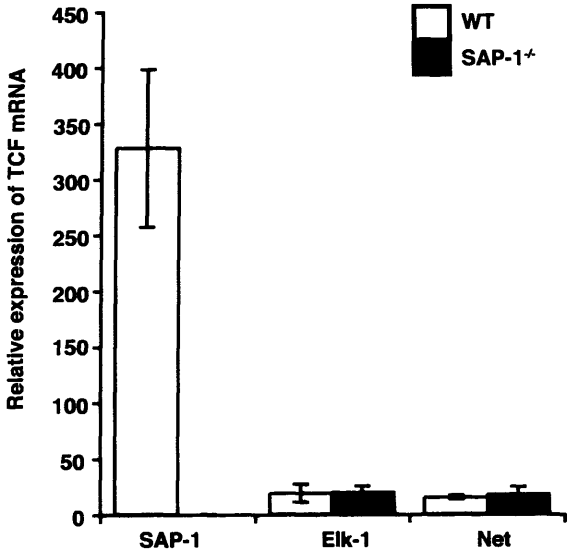
Figure 3.11. TCF mRNA expression in thymocyte sub-populations. Real time RT-PCR was used to assess the relative contribution and expression of the three TCFs within individual thymocyte populations all normalised to HPRT expression. HPRT expression was equivalent in all populations analysed. **A.** Relative expression of the three TCF mRNAs. **B.** Relative contributions of the three TCF mRNAs to total TCF mRNA.

T_{regs}. To address this question the expression of the three TCFs was examined by real-time RT-PCR in WT T_{regs} and compared with the other thymocyte populations. WT thymocytes were sorted into the following subpopulations: Single positive $CD4^+$ and $CD8^+$ (SP) T cells, double negatives $CD4^-CD8^-$ (DN), double positives $CD4^+CD8^+$(DP), and regulatory thymocytes ($CD4^+CD25^+$). RNA was extracted, reversed transcribed and then the cDNA was used as target DNA in a real-time PCR reaction. The relative expression level of the three TCF mRNAs was similar between the various subpopulations with SAP-1 being most highly expressed followed by Net and then Elk-1. Intriguingly expression in the double negative subpopulation was reduced (Figure 3.11A). This raises an interesting possibility that the TCFs may be differentially expressed during thymocyte development between the DN stage and DP stage. Analysis of the relative contribution of the three TCFs was similar to that previously determined for DP thymocytes by RNase protection (Costello et al., 2004). For all the thymocyte cell populations examined, including T_{regs}, SAP-1 was the predominant TCF contributing approximately 80% of the TCF mRNA found in the cells with Net contributing approximately 15% and Elk-1 contributing the remaining 5% (Figure 3.11B). Thus regulatory T cells expressed the TCFs at similar levels to other DP and SP T cell populations.

3.9.2 Expression of Elk-1 and Net do not compensate for the loss of SAP-1 mRNA

One possible explanation for the apparent SAP-1 independence of T_{reg} development is that the remaining TCFs may up-regulate their mRNA and compensate for the loss of SAP-1. To address this DP thymocytes were sorted from WT and SAP-$1^{-/-}$ animals and assessed for mRNA expression of the three TCFs. The levels of Elk-1 and Net mRNA remained unaltered in SAP-$1^{-/-}$ DP thymocytes (Figure 3.12A). A similar result was obtained when WT and SAP-$1^{-/-}$ $CD4^+CD25^+$ thymocytes were examined (Figure 3.12B). It is therefore unlikely that compensation by Elk-1 and Net is an explanation for normal T_{reg} development in SAP-$1^{-/-}$ animals.

A



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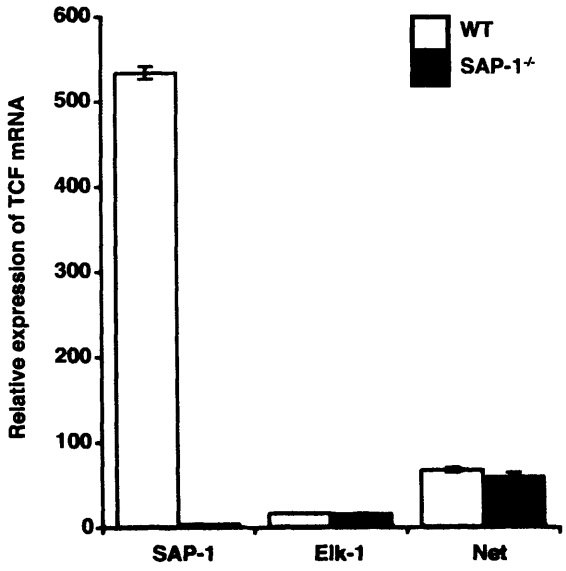
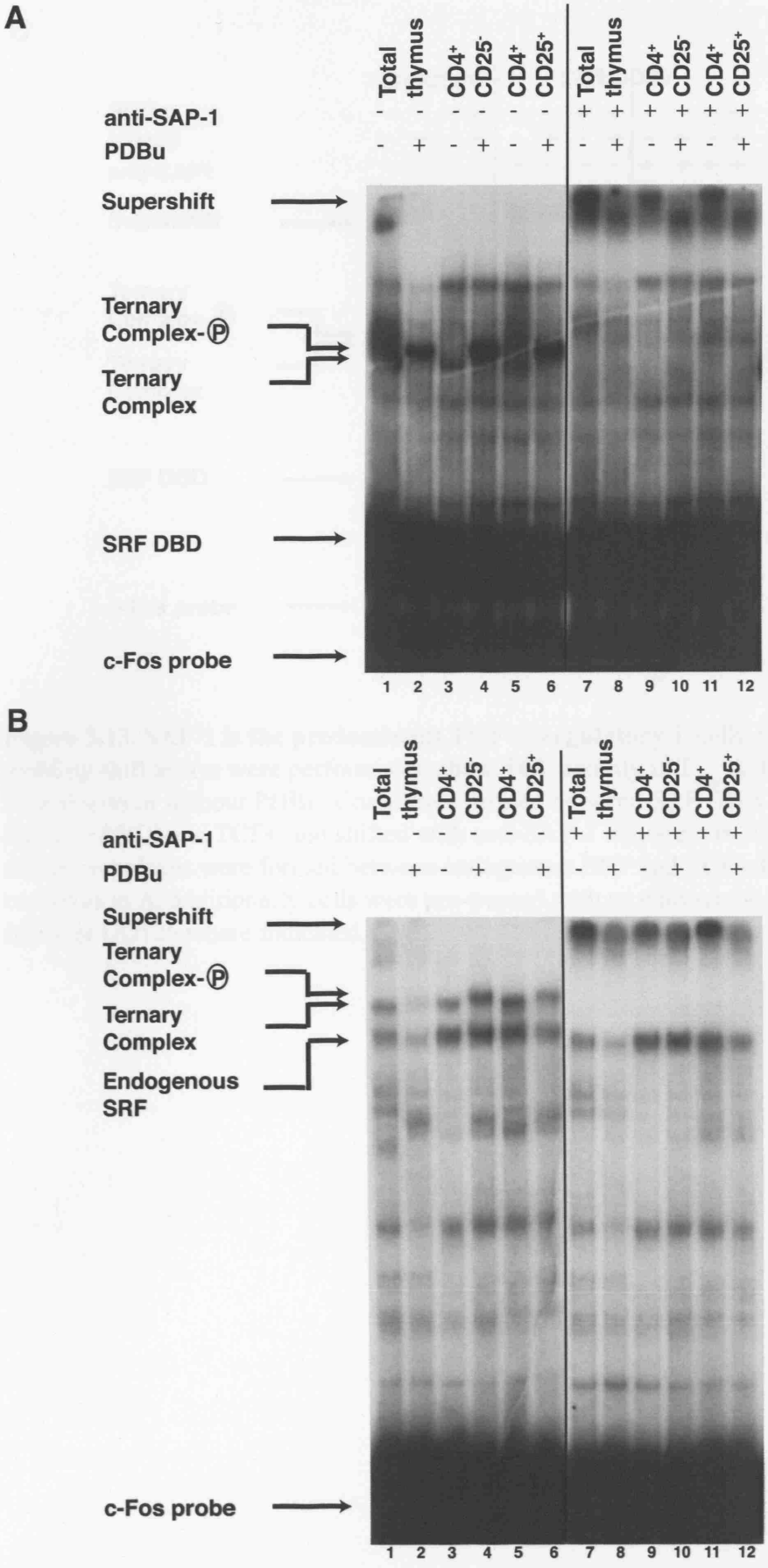


Figure 3.12. Elk-1 and Net mRNA does not increase in SAP-1$^{-/-}$ cells. Real time RT-PCR was used to determine the relative expression of the three TCF mRNAs in WT and SAP-1$^{-/-}$ cells. Expression was normalised to HPRT expression which was equivalent in all samples examined. **A.** DP thymocytes **B.** CD4^{+}CD25^{+} thymocytes. Results represent three separate RNA samples.

3.10 TCF activity in Regulatory T cells

To assess TCF activity at the biochemical level I carried out gel mobility shift assays on different T cell populations. Gel mobility shift assays have been used previously to demonstrate TCF activity in DP thymocytes (Costello et al., 2004). These assays involve the preparation of nuclear cell extract which is then incubated with a radioactive DNA probe generated from the c-Fos promoter containing the serum response element (SRE) (Marais et al., 1993). The TCFs have been shown to bind to DNA in the presence of serum response factor (Shaw et al., 1989). These experiments can either be performed with endogenous SRF present in the nuclear extracts or by adding the SRF DNA binding domain (SRF residues 133-265). Addition of the SRF DNA binding domain enhances the sensitivity of the assay, as the complex runs faster thus mobility changes are easier to detect (Marais et al., 1993). Addition of the SRF DNA binding domain also increases the yield by driving complex formation, as these reactions are not performed at saturating levels. Any complexes formed can be detected by running on a non-denaturing polyacrylamide gel and then subjecting it to autoradiography. Additionally antibodies can be added to the reaction to identify components of the complexes, if a particular protein is present then the antibody will bind producing a more slowly migrating complex. This technique has been able to show complexes formed between SAP-1 and Elk-1 and under certain circumstances Net (Giovane et al., 1994; Price et al., 1995).

Due to the requirement for a minimum of $1x10^6$ cells for each experiment, to obtain sufficient nuclear extract peripheral regulatory T cells (sorted as $CD4^+CD25^+$ T cells) were compared with peripheral $CD4^+CD25^-$ T cells. Total thymus extract was included as a positive control as this has previously been shown to form ternary complexes between SAP-1 and SRF (Costello et al., 2004). Initial experiments were performed with the inclusion of the SRF DNA binding domain. It was possible to detect complexes in all samples, including peripheral $CD4^+CD25^+$ T cells (Figure 3.13A lanes 5, and 6). The complexes were more apparent in cells that had been treated with phorbol dibutyrate (PDBu) (Figure 3.13A lanes 2, 4, and 6). PDBu results in the phosphorylation of the TCFs thereby resulting in a slower-moving complex in these gels.


A
Total thymus
CD4+ CD25-
CD4+ CD25+
Total thymus
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CD4+ CD25+
anti-SAP-1
PDBu
Supershift
Ternary Complex-Ⓟ
Ternary Complex
SRF DBD
c-Fos probe
1 2 3 4 5 6 7 8 9 10 11 12
B
Total thymus
CD4+ CD25-
CD4+ CD25+
Total thymus
CD4+ CD25-
CD4+ CD25+
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C

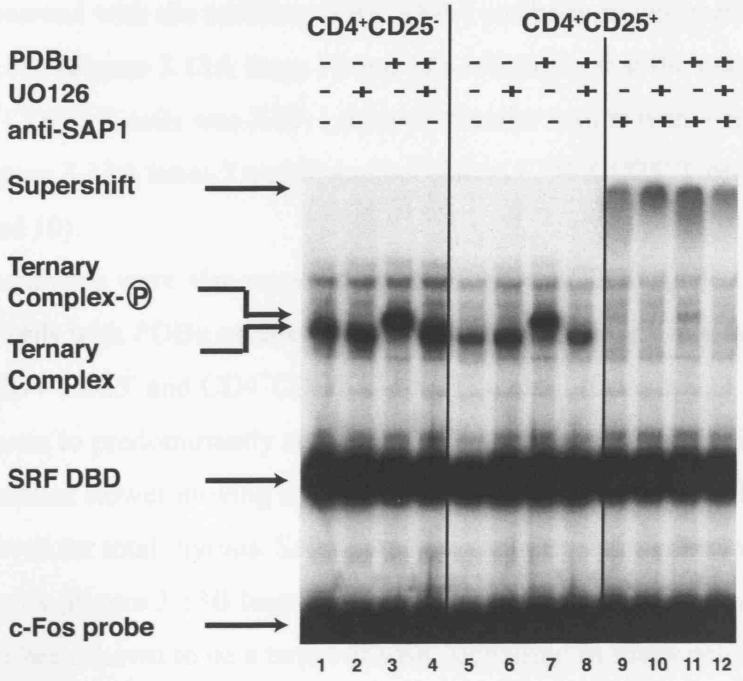


Figure 3.13. SAP-1 is the predominant TCF in regulatory T cells. Gel mobility shift assays were performed to show TCF activity in T_{regs} **A.** Cells treated with or without PDBu. Complexes formed between SRF DNA binding domain (DBD) and TCFs, and shifted with anti-SAP-1 antibody. **B.** As for **A.** except complexes were formed between endogenous SRF and TCFs. **C.** Cells treated as in **A.** additionally cells were pre-treated with or without the MEK inhibitor UO126 where indicated.

The ternary complexes could be shifted by incubation with anti-SAP-1 (Figure 3.13A lanes 7-12). As previously shown for total thymus, nearly a complete shift of the complex was observed with the addition of the SAP-1 antibody in peripheral $CD4^+CD25^+$ T cells (Figure 3.13A lanes 11 and 12), indicating that the majority of TCF activity in $CD4^+CD25^+$ T cells was SAP-1 derived. Similar results were observed for total thymus (Figure 3.13A lanes 7 and 8) and peripheral $CD4^+CD25^-$ T cells (Figure 3.13A lanes 9 and 10).

These experiments were also repeated with endogenous SRF. As described above the treatment of cells with PDBu resulted in a more defined complex in total thymus and peripheral $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells (Figure 13B lanes 2, 4 and 6). This complex was shown to predominantly consist of SAP-1 by the addition of SAP-1 antibody and resultant slower moving complex (Figure 3.13B lanes 7-12). Thus as previously observed for total thymus, SAP-1 was shown to be the predominant TCF in $CD4^+CD25^+$ T cells (Figure 3.13B lanes 11 and 12) consistent with the RNA data.

SAP-1 had been shown to be a target of ERK signalling in many cell types including DP thymocytes (Costello et al., 2004). To confirm that this was also the case for regulatory T cells, gel mobility assays were performed with cells pre-treated with the MEK inhibitor UO126. As previously shown treatment of $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells with PDBu resulted in a slower moving complex (Figure 3.13C lanes 3 and 7). When these cells were additionally pre-treated with the MEK inhibitor UO126 this slower moving complex was no longer detected (Figure 3.13C lanes 4 and 8), indicating it was induced by ERK signalling. Thus SAP-1 is not only active in regulatory T cells in that it is capable of forming complexes with its transcription partner SRF and DNA containing SRF binding sites, it is also subjected to similar activation through ERK signalling as has been demonstrated for conventional T cells.

3.11 Summary

Data presented in this chapter highlight fundamental differences in the development of $CD4^+Foxp3^-$ and $CD4^+Foxp3^+$ T cell populations. Despite the severe defect in positive selection of $CD4^+$ and $CD8^+$ SP thymocytes in SAP-$1^{-/-}$ animals, examination of the $CD4^+CD25^+$ T cell population revealed no such developmental defect. Refinement of regulatory T cell identification using other markers including the lineage specific marker Foxp3 confirmed that the generation of these cells was

unperturbed by the loss of SAP-1. Furthermore the data indicates that as in other thymocyte subsets, SAP-1 is the predominant TCF in regulatory T cells and its loss is not compensated by increased expression of remaining family members. It is unclear as to why T_{reg} development is unaffected by the loss of SAP-1. One possibility is that T_{reg} development has a lower requirement for TCF signalling than conventional $CD4^+$ T cells; alternatively T_{reg} development may be a TCF independent process.

4 Results – T_{reg} development in animals deficient in other components of the Serum Response Factor (SRF) pathway

4.1 Abstract

SAP-$1^{-/-}$ mice have a severe defect in positive selection. The other members of the TCF family of transcription factors have not been reported to have an immune phenotype (Cesari et al., 2004; Ayadi et al., 2001). Regulatory T cell development is independent of SAP-1; however it remained possible that the other TCFs have a specific role in regulatory T cell development. This was addressed in single and multiple TCF deficient mice. It was found that there was no specific role for either Net or Elk-1 in T_{reg} development. Deletion of multiple TCFs resulted in an exacerbated defects in positive selection, however reduced numbers of T_{regs} was only detected in animals triply deficient for the TCFs. The transcription partner of the TCFs the serum response factor (SRF) was also assessed for a possible role in regulatory T cell development. SRF deficient mice have previously been reported to be defective in positive selection (Fleige et al., 2007). Analysis of regulatory T cell development in SRF deficient mice revealed that both positive selection and regulatory T cell development was blocked in these animals, indicating that SRF is required for T_{reg} development. Coupled with the data from mice triply deficient for the TCFs, there appears to be an SRF-dependent, TCF-independent process during positive selection which has not previously been observed.

4.2 Introduction

In Chapter 3 it has been demonstrated that thymic T_{reg} development was unperturbed in SAP-$1^{-/-}$ animals despite the fact that these mice displayed a severe

defect in positive selection. It is possible that the remaining TCF family members play a specific role in T_{reg} development. Alternatively it may be that there is a threshold of TCF activity that is required for T_{reg} generation and that this threshold is lower than that required for positive selection. One approach to address this possibility would be to delete the other TCF family members, Elk-1 and Net, and examine what impact this has on T_{reg} development, individually or in combination. Mice deficient in Elk-1 or deficient for Net activity have been generated by the Laboratories of A. Nordheim and B. Wasylyk respectively. A collaboration has been established between our laboratories to enable the generation of mice multiply deficient in the TCFs. Interestingly neither the Elk-$1^{-/-}$ or $Net^{\delta/\delta}$ mice have been reported to exhibit any immune phenotype in contrast to the SAP-$1^{-/-}$ mice (Cesari et al., 2004; Ayadi et al., 2001). The Elk-$1^{-/-}$ mice are generally healthy with a mild neurological defect. $Net^{\delta/\delta}$ mice were generated through the deletion of the Ets DNA binding domain resulting in a truncated Net protein called Netδ. This is likely to be a null allele, although the authors propose that it is a hypomorphic mutant, and results in early lethality. However analysis of these mice did not specifically examine T_{reg} development (personal communication). Thus it was possible that either Elk-1 or Net was specifically critical for T_{reg} development or that T_{reg} development required a threshold of TCF signalling. This chapter addresses the impact of multiple TCF deletion on T_{reg} development. Finally the transcription partner of the TCFs, the serum response factor (SRF), was also examined for a possible role in regulatory T cell development. Conditional deletion of SRF using the CD4-Cre demonstrated that SRF was required for positive selection, thus it may also be required for T_{reg} development. This was addressed through a collaboration with D. Degaelen, where SRF was deleted using the CD2-Cre.

4.3 $CD4^+CD25^+$ T cell development in Elk-$1^{-/-}$ and SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals

In collaboration with P. Costello and R. Nicolas within the laboratory, mice doubly deficient for SAP-1 and Elk-1 were generated. Thymocyte development was investigated in both Elk-$1^{-/-}$ and SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals. Consistent with published data, thymocyte development appeared unperturbed by the loss of Elk-1 as assessed by

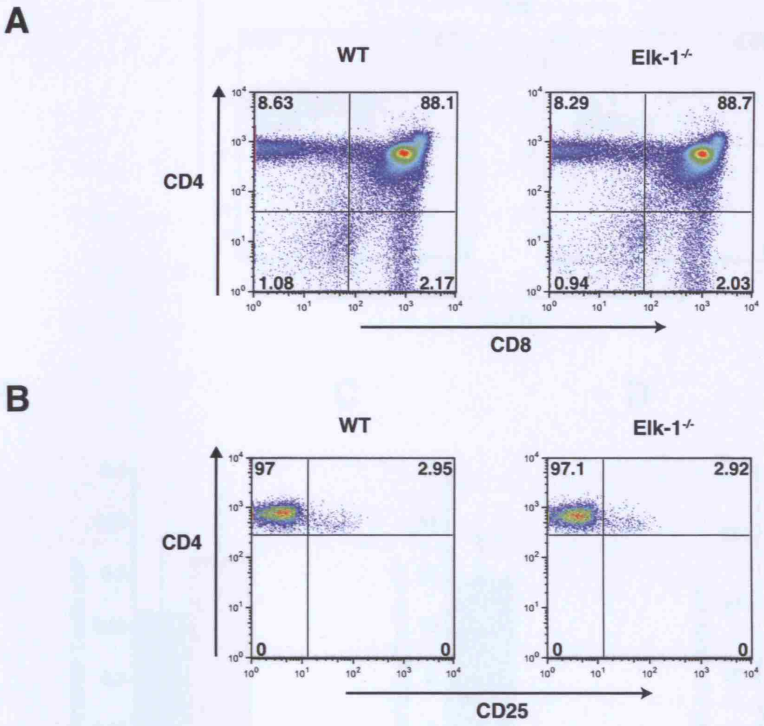


Figure 4.1. $CD4^+CD25^+$ T cell development is unaffected in Elk-$1^{-/-}$ animals. A. Thymocyte development was assessed by flow cytometry analysis of the expression of CD4 and CD8. **B**. Regulatory T cell development was assessed by the expression of CD25 on $CD4^+$ gated thymocytes.

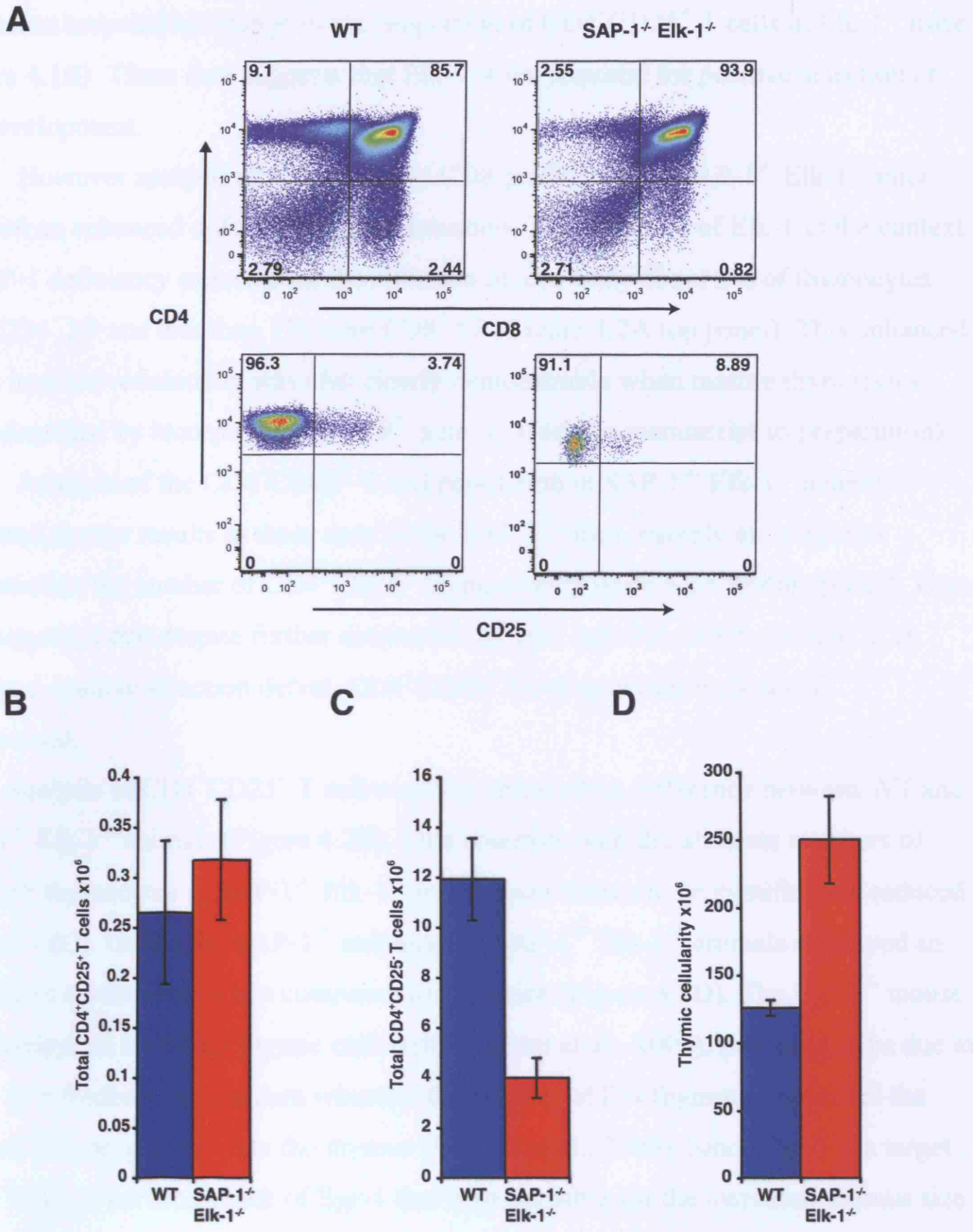


Figure 4.2. Normal numbers of $CD4^+CD25^+$ T cells develop in SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals. A. Thymocyte development assessed by flow cytometry analysis of CD4 and CD8 expression (top panels) and proportion of $CD25^+$ T cells on $CD4^+$ gated cells (bottom panels). **B.** Absolute numbers of $CD4^+CD25^+$ thymocytes in WT and SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals. **C.** Absolute numbers of $CD4^+CD25^-$ thymocytes in WT and SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals. **D.** Thymic cellularity.

expression of CD4 and CD8 (Figure 4.1A). Analysis of $CD4^+$ SP thymocytes for CD25 expression revealed no change in the proportion of $CD4^+CD25^+$ T cells in Elk-$1^{-/-}$ mice (Figure 4.1B). These data suggests that Elk-1 is not required for positive selection or T_{reg} development.

However analysis of thymic CD4 / CD8 profiles in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ mice revealed an enhanced defect in positive selection. Thus deletion of Elk-1 in the context of SAP-1 deficiency exacerbated the selection defect. Only about 2% of thymocytes were $CD4^+$ SP and less than 1% were $CD8^+$ SP (Figure 4.2A top panel). This enhanced defect in positive selection was also clearly demonstrable when mature thymocytes were identified by incorporating a TCR^{hi} gate (P. Costello manuscript in preparation).

Analysis of the $CD4^+CD25^+$ T cell population in SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals generated similar results to those seen in the SAP-$1^{-/-}$ mice, namely an increased proportion in the number of $CD4^+CD25^+$ thymocytes (Figure 4.2A bottom panel). These data suggested that despite further diminution of TCF activity, which resulted in an enhanced positive selection defect, $CD4^+CD25^+$ T cell generation remained unperturbed.

Analysis of $CD4^+CD25^+$ T cell numbers revealed no difference between WT and SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals (Figure 4.2B). This contrasts with the absolute numbers of $CD4^+$ SP thymocytes in SAP-$1^{-/-}$ Elk-$1^{-/-}$ which were shown to be significantly reduced (Figure 4.2C). Unlike the SAP-$1^{-/-}$ animals, the SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals displayed an increase in thymus size when compared to WT mice (Figure 4.2D). The Egr-$1^{-/-}$ mouse also displays an increased thymic cellularity (Bettini et al., 2002), proposed to be due to a lack of a feedback mechanism whereby the number of DN thymocytes control the entry of new progenitors into the thymus (Schnell et al., 2006). Since Egr-1 is a target of the TCFs, it perhaps a lack of Egr-1 that is responsible for the increased thymus size observed in SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals.

The increased thymus size in SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals may have been expected to result in an increased number of T_{regs} if their development was unaffected. However studies with transgenic mice have led to the proposal that T_{reg} development occurs in a two-step model of which the second step involves a limited signal and as such development of only a certain number of T_{regs} can be supported (reviewed in Liston and Rudensky, 2007). If this model is correct, then even with the increased thymus size an increase in the number of T_{regs} would not be expected and thus these data would indicate that T_{reg} development is independent of SAP-1 and Elk-1. The implications of

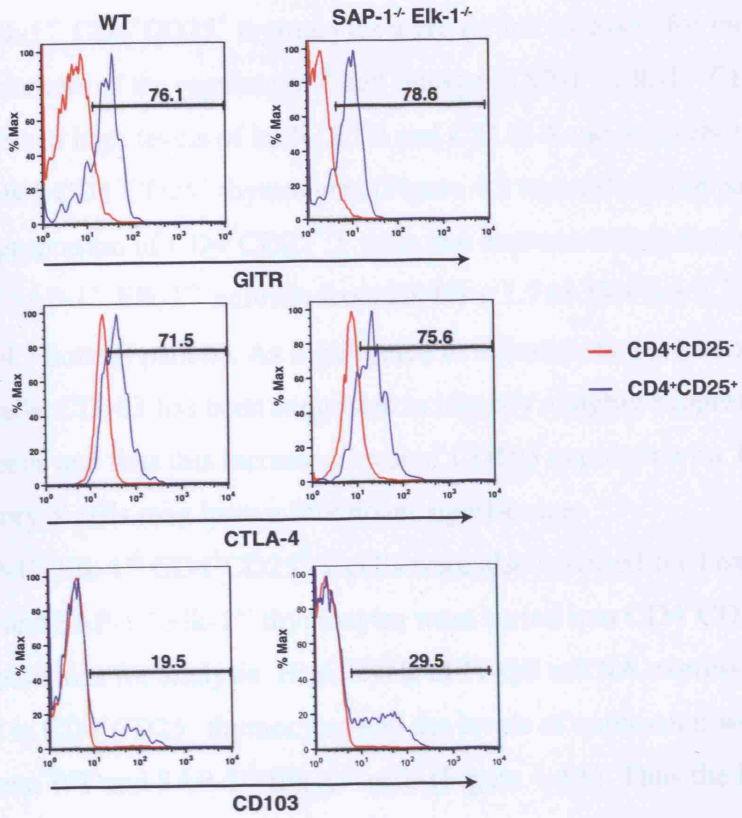


Figure 4.3. SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4^{+}CD25^{+} thymocytes express regulatory markers. Expression of regulatory markers was assessed by flow cytometry analysis. GITR - top panels, CTLA-4 - middle panels, and CD103 - bottom panels.

equivalent numbers of T_{regs} being detected in WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals will be explored further in the discussion.

4.4 SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4^{+}CD25^{+} T cells express other regulatory markers including Foxp3.

SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4^{+}CD25^{+} thymocytes were further assessed for the expression of other known markers of the regulatory T cell lineage. SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4^{+}CD25^{+} thymocytes expressed high levels of both GITR and CTLA-4 and at levels equivalent to that observed in WT CD4^{+}CD25^{+} thymocytes (Figure 4.3 top and middle panels). Intriguingly the proportion of CD4^{+}CD25^{+} T cells that expressed high levels of CD103 was increased in SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals from 23.4% ± 1.7 to 33.4% ± 5.3 (n=3, p=0.04) (Figure 4.3 bottom panels). As highlighted in reference to peripheral SAP-1$^{-/-}$ CD4^{+}CD25^{+} T cells, CD103 has been suggested to identify a highly suppressive subset of regulatory T cells and thus this increased level of CD103 expression on TCF deficient regulatory T cells may have a functional significance.

The SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4^{+}CD25^{+} T cells were also assessed for Foxp3 expression. WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ thymocytes were sorted into CD4^{+}CD25^{-} or CD4^{+}CD25^{+} populations for analysis. High levels of Foxp3 mRNA expression could only be detected in CD4^{+}CD25^{+} thymocytes and the levels of expression were equivalent between WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ cells (Figure 4.4A). Thus the loss of Elk-1 even in combination with SAP-1 deficiency did not affect Foxp3 expression. Moreover intracellular staining revealed that approximately 88% of SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4^{+}CD25^{+} thymocytes expressed high levels of Foxp3 protein and at a level equivalent to that observed in WT cells (Figure 4.4B top panels). Likewise when SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4^{+}CD25^{+} T cells from spleen and lymph nodes were examined more than 85% of the cells expressed Foxp3 at a level equivalent to WT (Figure 4.4B middle and bottom panels).

Examination of thymocyte profiles incorporating intracellular Foxp3 staining reflected data collected for the development of CD4^{+}CD25^{+} T cells confirming that equivalent numbers of regulatory T cells develop in WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals. The proportion of CD4^{+}Foxp3^{+} thymocytes was significantly increased in the

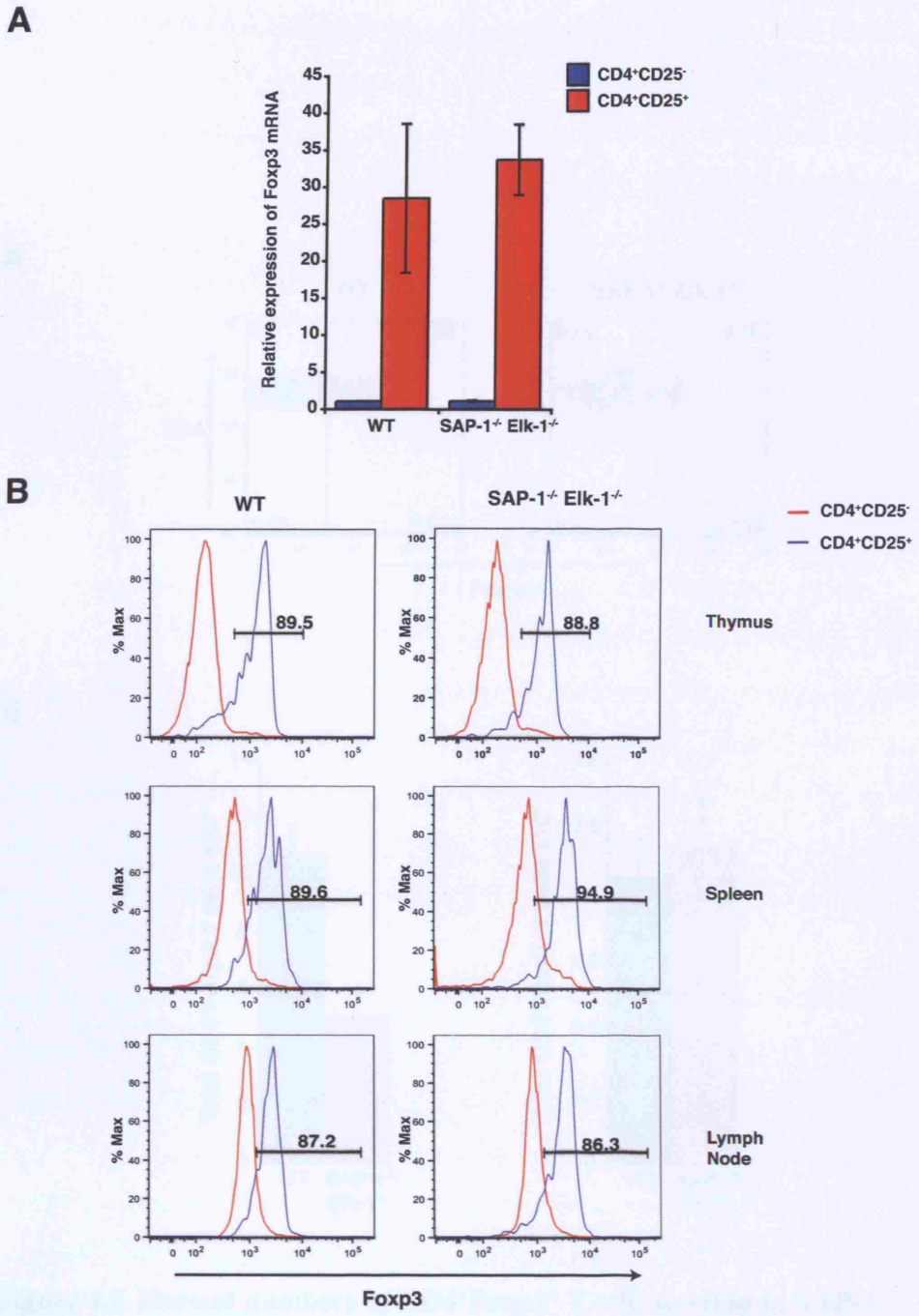


Figure 4.4. SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4^{+}CD25^{+} T cells express Foxp3. A. Cells were sorted into CD4^{+}CD25^{-} and CD4^{+}CD25^{+} T cell populations from WT and SAP-1$^{-/-}$Elk-1$^{-/-}$ animals and examined for Foxp3 mRNA expression by real-time RT-PCR normalised to GAPDH. GAPDH expression was equivalent in all samples examined. Three separate RNA preparations were examined in this way. Relative expression in WT CD4^{+}CD25^{-} T cells was given an arbitrary value of 1. **B.** Foxp3 expression was assessed on a per cell basis by intracellular staining. The majority of both WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4^{+}CD25^{+} T cells express Foxp3. Cells isolated from: thymus (top panels); spleen (middle panels); and lymph node (bottom panels).

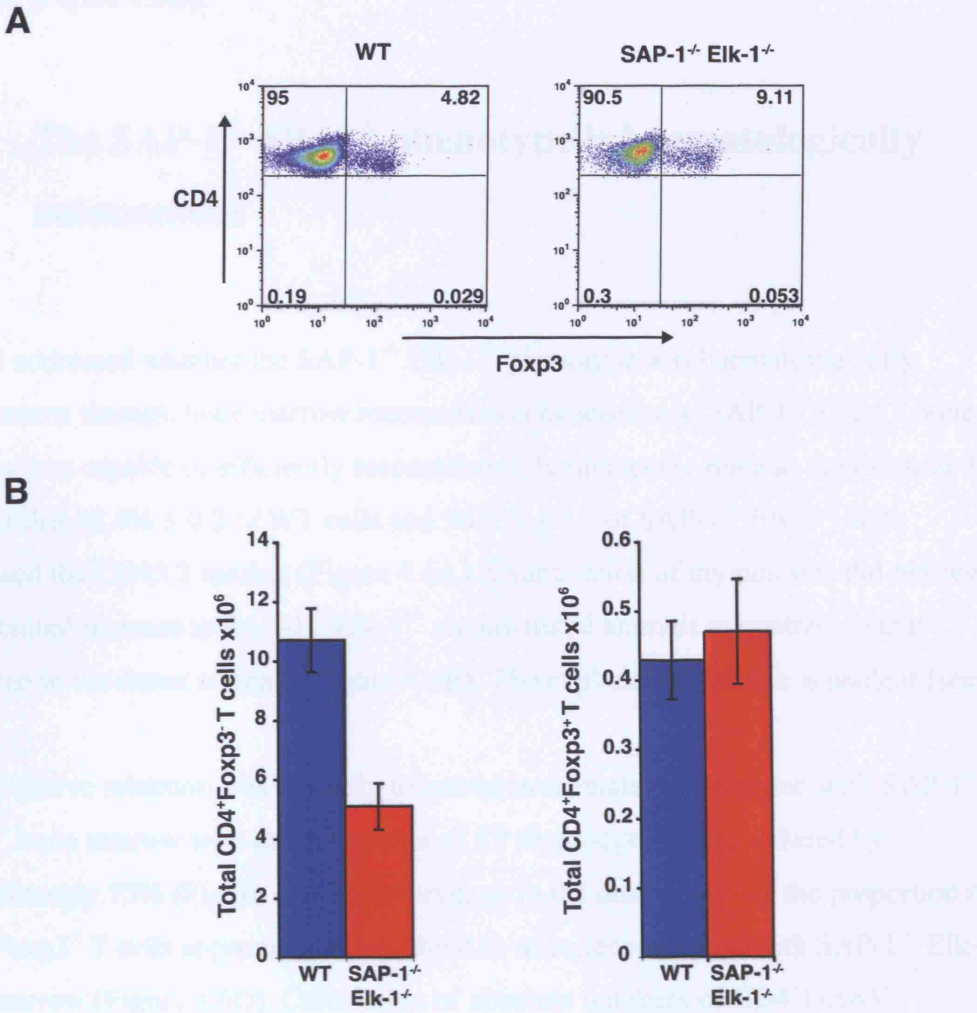


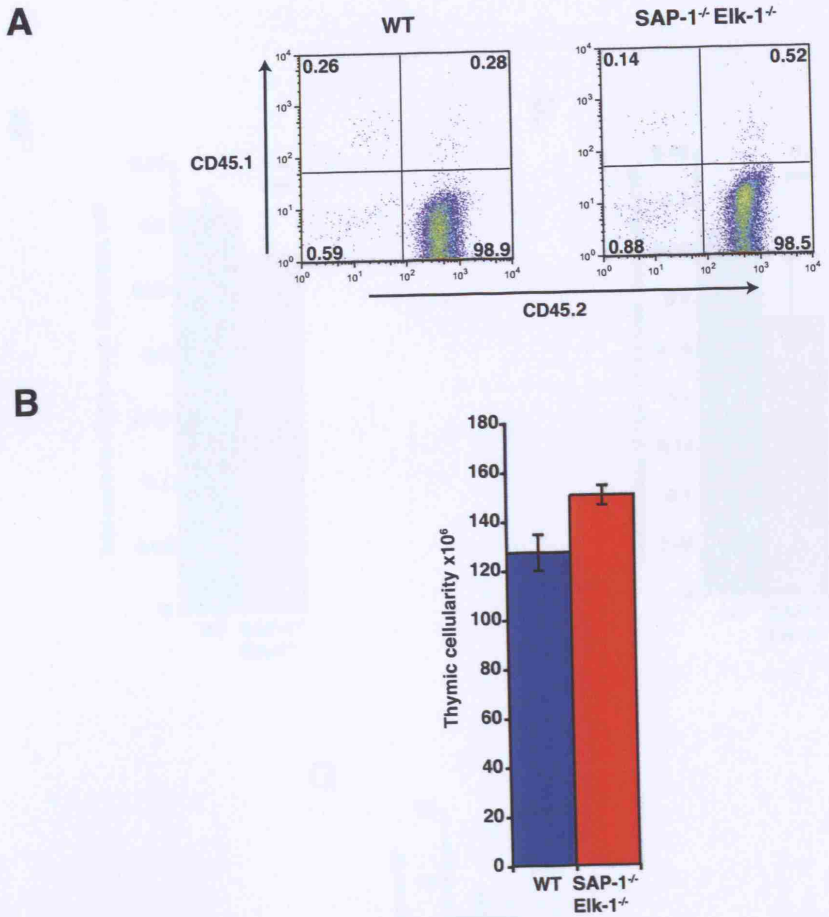
Figure 4.5. Normal numbers of CD4$^+$Foxp3$^+$ T cells develop in SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals. A. Proportion of Foxp3$^+$ T cells on CD4$^+$ gated cells. **B.** Absolute numbers of CD4$^+$Foxp3$^-$ (left) and CD4$^+$Foxp3$^+$ thymocytes (right) in WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals.

SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals from 3.5% ± 0.3 in WT animals to 7.4% ± 0.3 in SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals (n=6, $p < 0.0001$) (Figure 4.5A). The absolute numbers of $CD4^+Foxp3^+$ thymocytes were calculated. As described above the thymus data is complicated by the increased thymus size however, despite a severe reduction in the number of total $CD4^+$ SP thymocytes in SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals the number of $CD4^+Foxp3^+$ T cells was not affected (Figure 4.5B).

4.5 The SAP-$1^{-/-}$ Elk-$1^{-/-}$ phenotype is haematologically autonomous

I addressed whether the SAP-$1^{-/-}$ Elk-$1^{-/-}$ phenotype was haematologically autonomous through bone marrow reconstitution experiments. SAP-$1^{-/-}$ Elk-$1^{-/-}$ bone marrow was capable of efficiently reconstituting lymphopenic mice as demonstrated by the fact that 98.4% ± 0.2 of WT cells and 98.5% ± 0.1 of SAP-$1^{-/-}$ Elk-$1^{-/-}$ cells expressed the CD45.2 marker (Figure 4.6A). Examination of thymus size did not reveal a substantial increase in SAP-$1^{-/-}$ Elk-$1^{-/-}$ reconstituted animals in contrast to that observed in the donor animals (Figure 4.6B). The explanation for this is unclear (see 7.5.1).

Positive selection was severely impaired in animals reconstituted with SAP-$1^{-/-}$ Elk-$1^{-/-}$ bone marrow with the proportion of SP thymocytes being reduced by approximately 75% (Figure 4.6C). However as in the donor animals the proportion of $CD4^+Foxp3^+$ T cells approximately doubled in mice reconstituted with SAP-$1^{-/-}$ Elk-$1^{-/-}$ bone marrow (Figure 4.6D). Calculation of absolute numbers of $CD4^+Foxp3^+$ thymocytes revealed no difference between mice reconstituted with SAP-$1^{-/-}$ Elk-$1^{-/-}$ bone marrow and WT bone marrow (Figure 4.6E). This was also true when absolute numbers were calculated with the TCR^{hi} gate applied (Figure 4.6F). In contrast calculation of absolute numbers of $CD4^+$ SP thymocytes revealed a significant reduction in animals reconstituted with SAP-$1^{-/-}$ Elk-$1^{-/-}$ bone marrow (Figure 4.6G). Thus these data, along with that from donor animals, would suggest that the loss of both SAP-1 and Elk-1 results in a substantial defect in positive selection, which is a haematologically autonomous phenotype. In contrast neither the loss of Elk-1 or SAP-1, either individually or in combination, resulted in a reduction in the numbers of regulatory T cells that develop in these animals.


A
WT
SAP-1-/- Elk-1-/-
0.26
0.28
0.59
98.9
0.14
0.52
0.88
98.5
CD45.1
CD45.2
B
Thymic cellularity x10^6
180
160
140
120
100
80
60
40
20
0
WT
SAP-1-/- Elk-1-/-
C
WT
SAP-1-/- Elk-1-/-
9.05
86.2
2.24
2.54
2.79
93.5
2.13
1.57
CD4
CD8
D
WT
SAP-1-/- Elk-1-/-
94.9
3.66
1.32
0.12
90.1
7.58
2.27
0.07
CD4
Foxp3

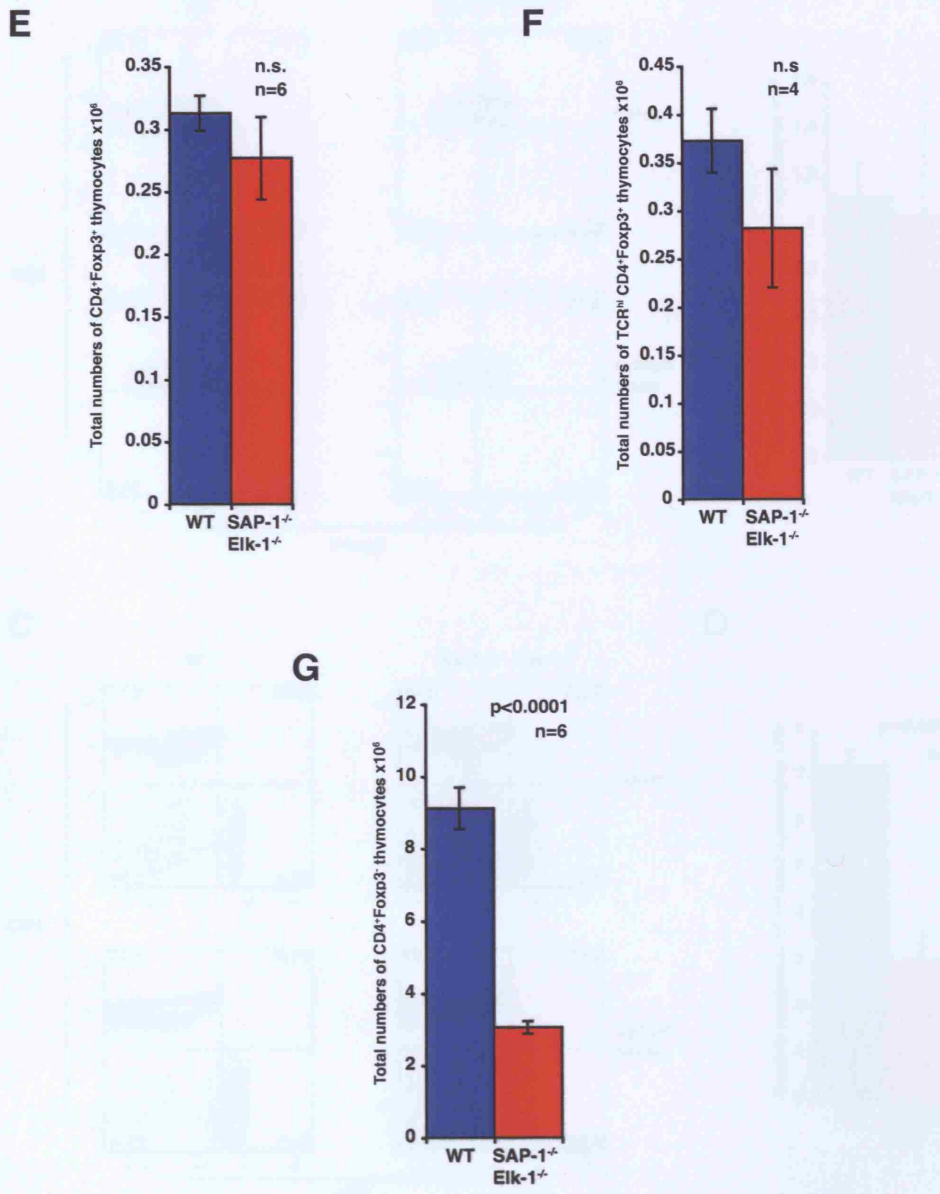


Figure 4.6. SAP-1$^{-/-}$ Elk-1$^{-/-}$ phenotype is haematologically autonomous. A. Efficiency of reconstitution was assessed by the expression of CD45.2 in mice reconstituted with WT or SAP-1$^{-/-}$ Elk-1$^{-/-}$ bone marrow. **B.** No change in thymic cellularity was detected in the SAP-1$^{-/-}$ Elk-1$^{-/-}$ reconstitutions. **C.** Thymocyte development assessed by flow cytometry analysis of CD4 and CD8 expression on CD45.2^{+} gated cells. **D.** Regulatory T cell development assessed by the expression of Foxp3 on CD45.2^{+}CD4^{+} gated cells. **E-G.** Absolute numbers were calculated for WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ reconstitutions **E.** CD4^{+}Foxp3^{+} thymocytes. **F.** TCR^{hi}CD4^{+}Foxp3^{+} thymocytes. **G.** CD4^{+}Foxp3^{-} thymoctytes.

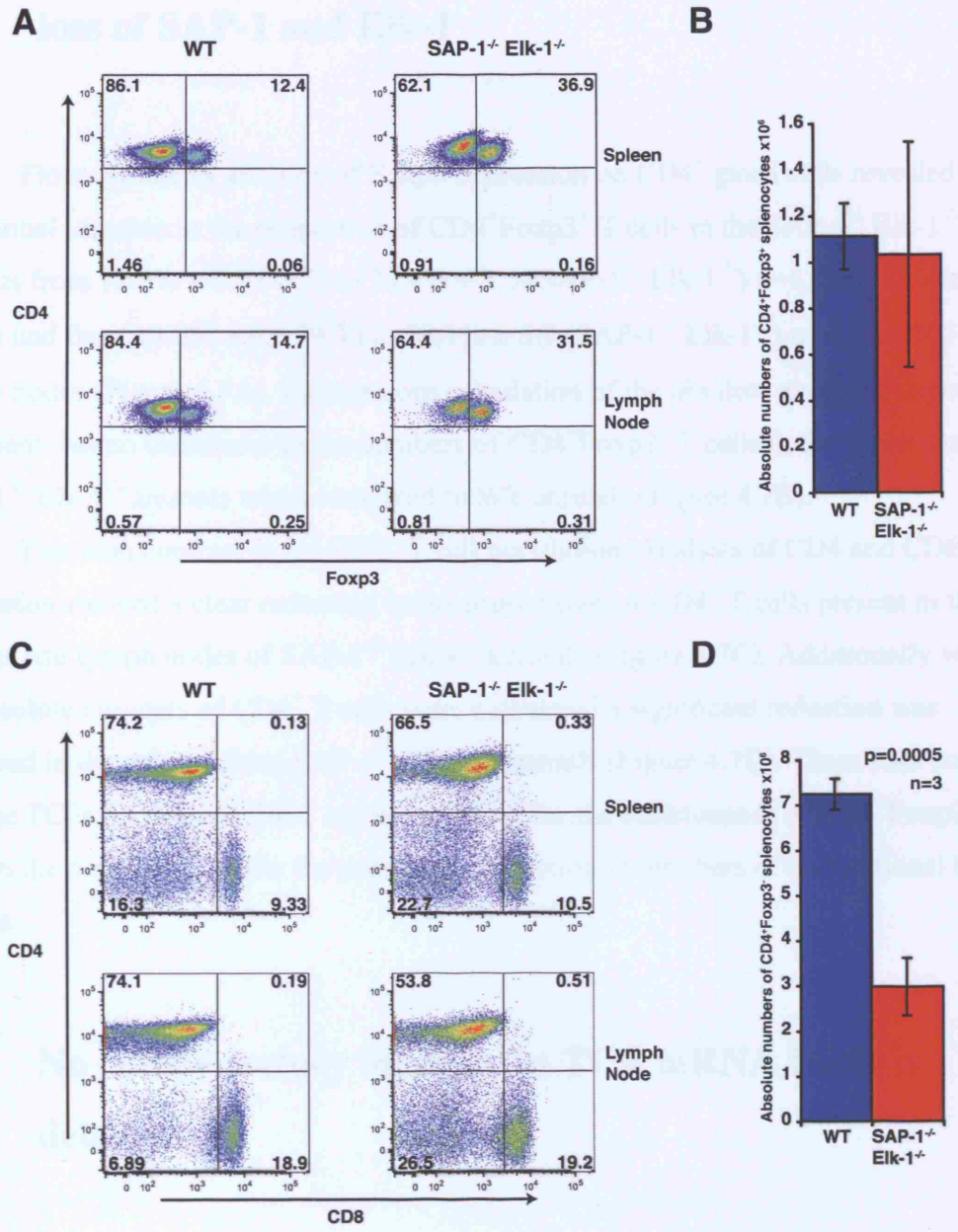


Figure 4.7. T_{reg} maintenance in the periphery is normal in SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals. A. Expression of Foxp3 examined on CD4$^+$ gated T cells in WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals. Cells isolated from: spleen (top panels); and lymph node (bottom panels). **B.** No reduction was observed in the numbers of CD4$^+$Foxp3$^+$ splenocytes in SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals (n=3). **C.** CD4 and CD8 expression on Thy1.2$^+$ gated cells in WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals. Cells isolated from: spleen (top panels); and lymph nodes (bottom panels). **D.** Numbers of CD4$^+$ T cells in SAP-1$^{-/-}$ Elk-1$^{-/-}$ spleens are significantly reduced.

4.6 Maintenance of T_{regs} in the periphery is unaffected by loss of SAP-1 and Elk-1

Flow cytometry analysis of Foxp3 expression on $CD4^+$ gated cells revealed a substantial increase in the proportion of $CD4^+Foxp3^+$ T cells in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals from 12.3% ± 0.9 (WT) to 34.4% ± 3.5 (SAP-$1^{-/-}$ Elk-$1^{-/-}$) n=4, $p<0.0001$ in the spleen and from 12.0% ± 0.6 (WT) to 29.1% ± 6.9 (SAP-$1^{-/-}$ Elk-$1^{-/-}$) n=4, $p<0.005$ in lymph nodes (Figure 4.7A). Furthermore calculation of the absolute numbers showed that there was no difference in the numbers of $CD4^+Foxp3^+$ T cells in the spleens of SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals when compared to WT animals (Figure 4.7B).

This is in contrast to the $CD4^+$ T cell population. Analysis of CD4 and CD8 expression showed a clear reduction in the proportions of $CD4^+$ T cells present in the spleens and lymph nodes of SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals (Figure 4.7C). Additionally when the absolute numbers of $CD4^+$ T cells were calculated a significant reduction was observed in the spleens from SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals (Figure 4.7D). These data suggest that the TCFs SAP-1 and Elk-1 are not required for the maintenance of $CD4^+Foxp3^+$ T cells in the periphery despite the substantial reduction in numbers of conventional $CD4^+$ T cells.

4.7 No compensatory increase in TCF mRNA levels is detected

The above results suggest that TCF signalling is not required for T_{reg} development; however it was possible that in the single and double TCF deficient animals the remaining TCFs were up-regulated in a compensatory manner. The levels of the three TCF mRNAs were analysed by real-time RT-PCR on cDNA prepared from sorted DP thymocytes. Upon depletion of SAP-1 the relative expression levels of both Elk-1 and Net remained constant. Furthermore no compensatory up-regulation of Net mRNA was observed upon deletion of both SAP-1 and Elk-1 (Figure 4.8A). Intriguingly there appeared to be a slight reduction in the levels of SAP-1 in the Elk-$1^{-/-}$ animals.

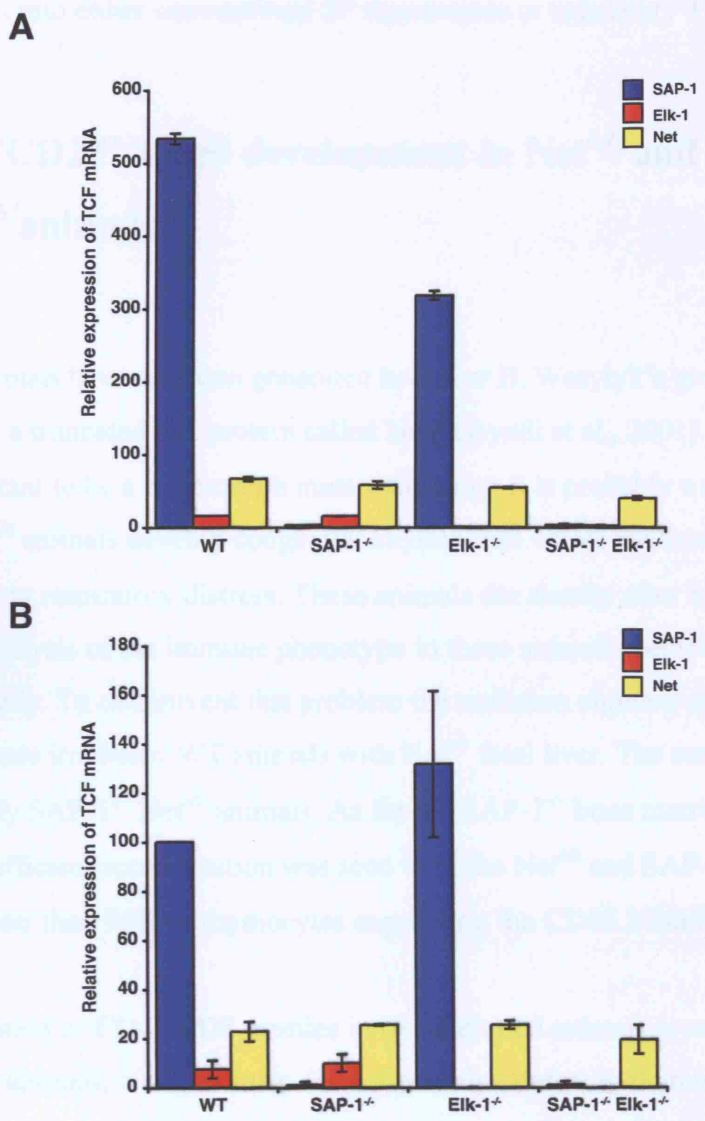


Figure 4.8. No compensatory increase in TCF mRNA levels. A. Relative expression of the three TCF mRNAs in sorted DP thymocytes normalised to GAPDH. GAPDH expression was equivalent in all samples examined. Analysis of triplicate samples, representative of three real-time RT-PCR experiments. **B.** Relative expression of the three TCF mRNAs in extracts prepared from total thymocytes. Expression of SAP-1 mRNA in WT cells was given an arbitary value of 100, expression of other TCFs and in other cells given relative to this. Data are the average of two separate mRNA samples analysed in triplicate.

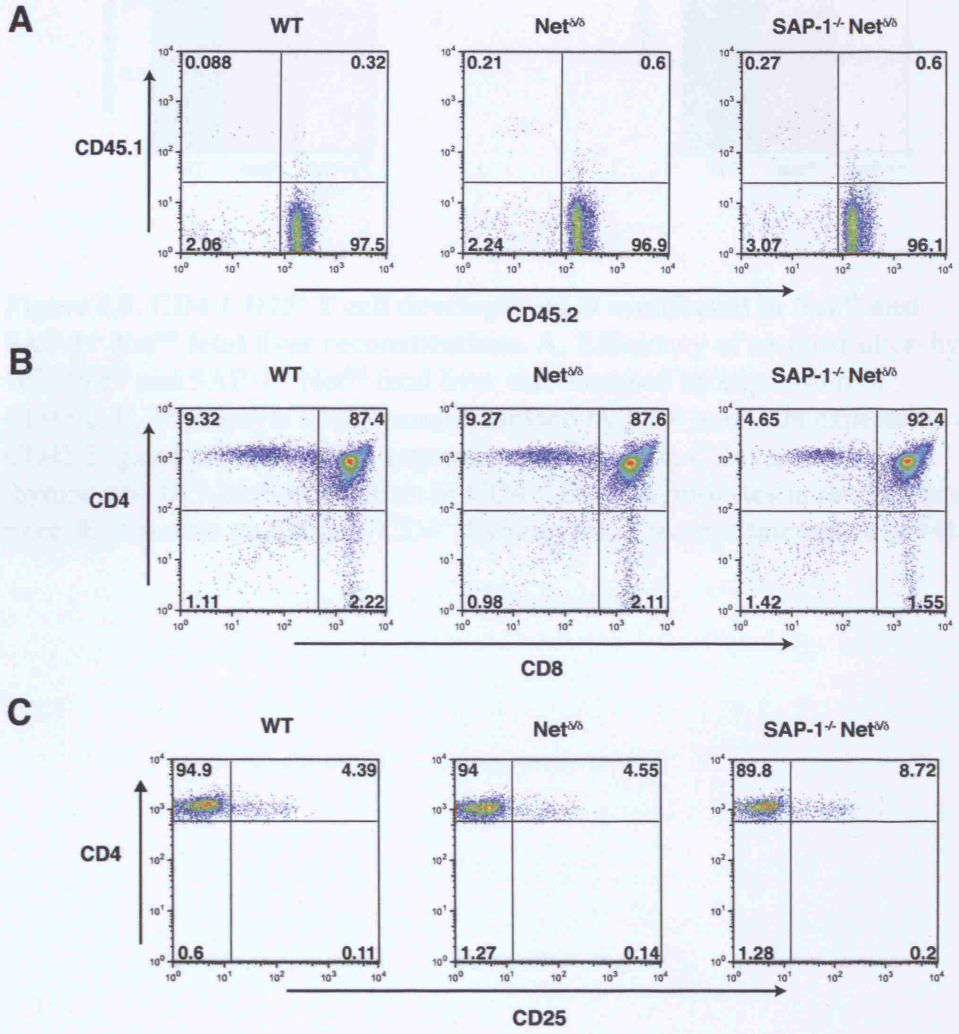
Analysis was also performed on cDNA from total thymus. Again no compensatory up-regulation of Net in SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals or Net and Elk-1 in SAP-1$^{-/-}$ animals was observed (Figure 4.8B). No reduction in SAP-1 mRNA was observed upon removal of Elk-1 alone. Thus it is unlikely that there is any mRNA compensation during the selection of cells into either conventional SP thymocytes or regulatory T cells.

4.8 CD4^{+}CD25^{+} T cell development in Net$^{\delta/\delta}$ and SAP-1$^{-/-}$ Net$^{\delta/\delta}$ animals

Net$^{-/-}$ animals have not been generated however B. Wasylyk's group generated mice expressing a truncated Net protein called Netδ (Ayadi et al., 2001). The authors propose this mutant to be a hypomorph mutant although it is probably a null mutation (see 7.5.1). Net$^{\delta/\delta}$ animals develop congenital chylothorax where the thoracic cavity fills with chyle causing respiratory distress. These animals die shortly after birth (Ayadi et al., 2001) and analysis of the immune phenotype in these animals therefore could not be undertaken directly. To circumvent this problem the radiation chimera approach was used to reconstitute irradiated WT animals with Net$^{\delta/\delta}$ fetal liver. The same approach was used to study SAP-1$^{-/-}$ Net$^{\delta/\delta}$ animals. As for the SAP-1$^{-/-}$ bone marrow reconstitutions efficient reconstitution was seen with the Net$^{\delta/\delta}$ and SAP-1$^{-/-}$ Net$^{\delta/\delta}$ fetal livers, with greater than 95% of thymocytes expressing the CD45.2 marker (Figure 4.9A).

Examination of CD4 / CD8 profiles in reconstituted animals revealed that as with the Elk-1$^{-/-}$ animals, Net$^{\delta/\delta}$ animals did not exhibit a defect in thymocyte development (Figure 4.9B middle panel). In contrast to the SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals however, Net$^{\delta/\delta}$ did not exacerbate the SAP-1$^{-/-}$ phenotype (Figure 4.9B right panel). This observation was also seen when mature thymocytes were identified by incorporating a TCR^{hi} gate (WT 8.6% ± 0.6; Net$^{\delta/\delta}$ 7.8% ± 0.2; SAP-1$^{-/-}$ Net$^{\delta/\delta}$ 4.0 ± 0.5). Thus Net does not appear to contribute (at least in the presence of Elk-1) to conventional SP thymocyte selection.

Examination of the proportions of CD4^{+}CD25^{+} T cells in Net$^{\delta/\delta}$ and the SAP-1$^{-/-}$ Net$^{\delta/\delta}$ thymi revealed no difference when compared with WT controls (Figure 4.9C). Analysis of cell numbers showed that the Netδ mutation did not affect T_{reg} development either alone or in combination with SAP-1$^{-/-}$ (Figure 4.9D). Furthermore analysis of


A
WT
Net^δ/δ
SAP-1^-/- Net^δ/δ
CD45.1
CD45.2
0.088
0.32
2.06
97.5
0.21
0.6
2.24
96.9
0.27
0.6
3.07
96.1
B
WT
Net^δ/δ
SAP-1^-/- Net^δ/δ
CD4
CD8
9.32
87.4
1.11
2.22
9.27
87.6
0.98
2.11
4.65
92.4
1.42
1.55
C
WT
Net^δ/δ
SAP-1^-/- Net^δ/δ
CD4
CD25
94.9
4.39
0.6
0.11
94
4.55
1.27
0.14
89.8
8.72
1.28
0.2

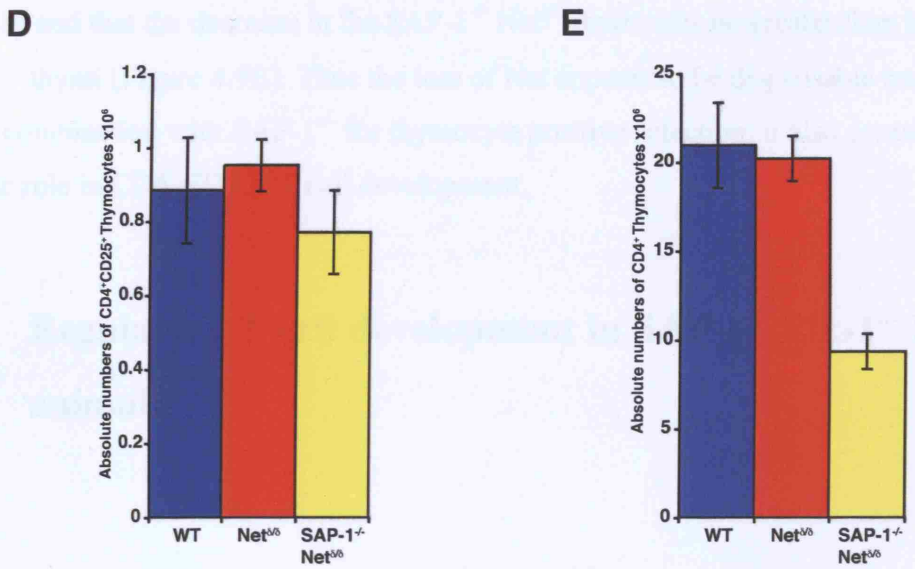


Figure 4.9. $CD4^+CD25^+$ T cell development is unaffected in $Net^{\delta/\delta}$ and $SAP\text{-}1^{-/-}$ $Net^{\delta/\delta}$ fetal liver reconstitutions. A. Efficiency of reconstitution by WT, $Net^{\delta/\delta}$ and $SAP\text{-}1^{-/-}$ $Net^{\delta/\delta}$ fetal liver was assessed by expression of CD45.2. **B.** Thymocyte development assessed by CD4 and CD8 expression on $CD45.2^+$ gated cells. **C.** CD25 expression analysed on $CD4^+$ gated thymocytes. **D.** Absolute numbers of $CD4^+CD25^+$ thymocytes in reconstituted mice. **E.** Absolute numbers of $CD4^+$ thymocytes in reconstituted mice (n=4)

absolute numbers of $CD4^+$ T cells in $Net^{\delta/\delta}$ thymi revealed no difference between WT controls, and that the decrease in the SAP-$1^{-/-}$ $Net^{\delta/\delta}$ thymi was no greater than in the SAP-$1^{-/-}$ thymi (Figure 4.9E). Thus the loss of Net appears to be dispensable both singly and in combination with SAP-$1^{-/-}$ for thymocyte positive selection; it also contributes no specific role in $CD4^+CD25^+$ T cell development.

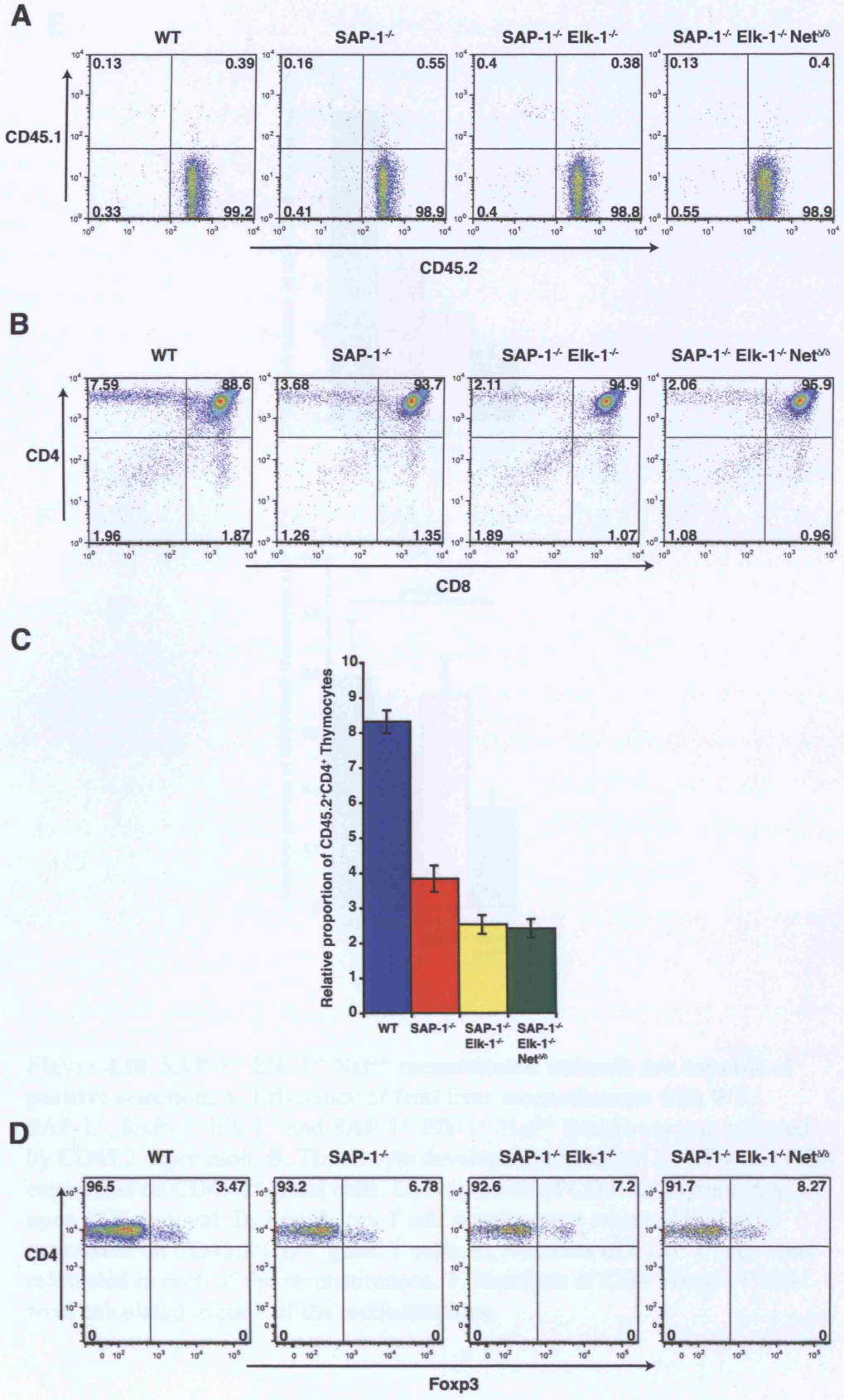
4.9 Regulatory T cell development in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals

4.9.1 Generation of SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals

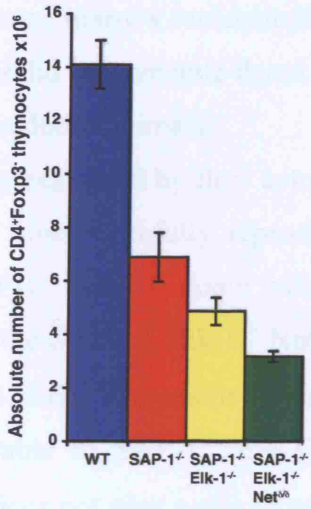
Finally, I addressed whether the combination of all three TCFs is required for regulatory T cell development. As outlined above due to the lethality seen in $Net^{\delta/\delta}$ animals we used fetal liver radiation chimeras. SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/-}$ males were crossed with SAP-$1^{-/-}$ Elk-$1^{+/-}$ $Net^{\delta/-}$ or SAP-$1^{+/-}$ Elk-$1^{-/-}$ $Net^{\delta/-}$ females which could potentially generate SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals. The $SAP^{-/-}$ $Elk^{+/-}$ $Net^{\delta/-}$ females were fertile producing litters although no SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals were present in these litters 2 weeks after birth. Timed matings were used to generate 14.5d embryos from which fetal livers could be harvested. After typing SAP-$1^{-/-}$, SAP-$1^{-/-}$ Elk-$1^{-/-}$, SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ and WT fetal livers were selected for reconstitution. Upon harvesting it was interesting to note that triple TCF deficient embryos were macroscopically indistinguishable from littermates. Irradiated B6.SJL mice were injected with fetal liver cells and analysed as before.

4.9.2 T_{reg} development in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ reconstitutions

Reconstitution efficiency was assessed by the expression of CD45.2, and as in previous reconstitutions, all genotypes efficiently reconstituted WT host animals with approximately 98% expressing CD45.2 (Figure 4.10A). No change in thymic cellularity


A
WT
SAP-1-/-
SAP-1-/- Elk-1-/-
SAP-1-/- Elk-1-/- Netδ/δ
CD45.1
CD45.2
0.13
0.39
0.33
99.2
0.16
0.55
0.41
98.9
0.4
0.38
0.4
98.8
0.13
0.4
0.55
98.9
B
WT
SAP-1-/-
SAP-1-/- Elk-1-/-
SAP-1-/- Elk-1-/- Netδ/δ
CD4
CD8
7.59
88.6
1.96
1.87
3.68
93.7
1.26
1.35
2.11
94.9
1.89
1.07
2.06
95.9
1.08
0.96
C
Relative proportion of CD45.2+CD4+ Thymocytes
WT
SAP-1-/-
SAP-1-/- Elk-1-/-
SAP-1-/- Elk-1-/- Netδ/δ
D
WT
SAP-1-/-
SAP-1-/- Elk-1-/-
SAP-1-/- Elk-1-/- Netδ/δ
CD4
Foxp3
96.5
3.47
93.2
6.78
92.6
7.2
91.7
8.27

E



F

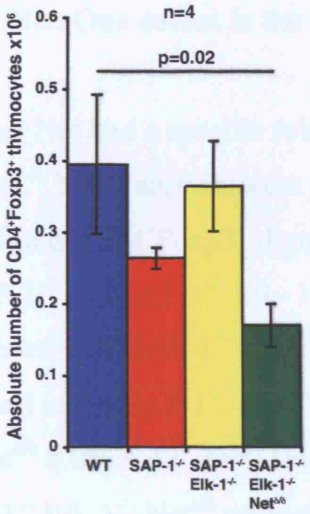


Figure 4.10. SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ reconstituted animals are capable of positive selection. A. Efficiency of fetal liver reconstitutions with WT, SAP-1$^{-/-}$, SAP-1$^{-/-}$ Elk-1$^{-/-}$ and SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ fetal livers was assessed by CD45.2 expression. **B.** Thymocyte development assessed by CD4 and CD8 expression on CD45.2^{+} gated cells. **C.** Proportion of CD4^{+} SP thymocytes upon TCF removal. **D.** Regulatory T cell development assessed by Foxp3 expression on CD45.2^{+}CD4^{+} gated T cells. **E.** Numbers of CD4^{+} T cells were calculated in each of the reconstitutions. **F.** Numbers of CD4^{+}Foxp3^{+} T cells were calculated in each of the reconstitutions.

was observed in any of the fetal liver reconstitutions (WT $237.7x10^6 \pm 17.8$; SAP-$1^{-/-}$ $230.8x10^6 \pm 20.4$; SAP-$1^{-/-}$ Elk-$1^{-/-}$ $210.6x10^6 \pm 20.8$; and SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ $226.6x10^6 \pm 17.6$). As for the bone marrow reconstitutions the animals reconstituted with SAP-$1^{-/-}$ Elk-$1^{-/-}$ fetal liver did not generate thymi that were double the size of WT, which had been observed in the donor animals.

T cell development was examined by flow cytometry analysis on $CD45.2^+$ gated cells. Reconstitution by fetal liver faithfully reproduced what had previously been observed in SAP-$1^{-/-}$ and SAP-$1^{-/-}$ Elk-$1^{-/-}$ donor animals. Significant numbers of SP thymocytes were observed in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ thymi with approximately 2% $CD4^+$ SP thymocytes (Figure 4.10B). The positive selection observed in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals was comparable to SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals (Figure 4.10C). This observation suggests that Net does not play a role in positive selection, even when other TCFs are absent. Furthermore the SP thymocytes present in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals are not due to the presence of Net. One caveat is the question over whether $Net^{\delta/\delta}$ is a null mutation (see 7.5.1).

Next I examined whether Net had a specific role in T_{reg} development. Thymocytes from SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals were examined for Foxp3 expression. As previously seen the proportion of $CD4^+Foxp3^+$ thymocytes substantially increased in animals reconstituted with SAP-$1^{-/-}$ and SAP-$1^{-/-}$ Elk- $1^{-/-}$ fetal livers; additionally the proportion in animals reconstituted with SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ fetal liver also increased to a level similar to that observed in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ thymus (SAP-$1^{-/-}$ Elk-$1^{-/-}$ $6.4\% \pm 0.4$, n=8; SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ $8.0\% \pm 1.0$, n=8) (Figure 4.10D). Thus T_{regs} were capable of developing in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ reconstitutions.

Analysis of absolute numbers of $CD4^+$ SP thymocytes repeated what had already been seen in donor animals in that there was a substantial reduction in numbers upon deletion of SAP-1 and Elk-1 (Figure 4.10E). Furthermore removal of the third TCF did result in a further reduction on top of that seen in animals reconstituted with SAP-$1^{-/-}$ Elk-$1^{-/-}$ fetal liver (SAP-$1^{-/-}$ Elk-$1^{-/-}$ $4.9x10^6 \pm 0.5$; and SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ $3.2x10^6 \pm 0.2$; n=8, p=0.01). The absolute numbers of $CD4^+Foxp3^+$ thymocytes was also determined. Reconstitution with SAP-$1^{-/-}$ or SAP-$1^{-/-}$ Elk-$1^{-/-}$ fetal liver did not result in any significant change in numbers (Figure 4.10F). When the numbers of $CD4^+Foxp3^+$ thymocytes were examined in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ fetal liver reconstitutions, a significant drop was observed although this drop was not as great as the reduction in total numbers of $CD4^+$ thymocytes (Figure 4.10F). These data may suggest that there is a requirement for TCF signalling.

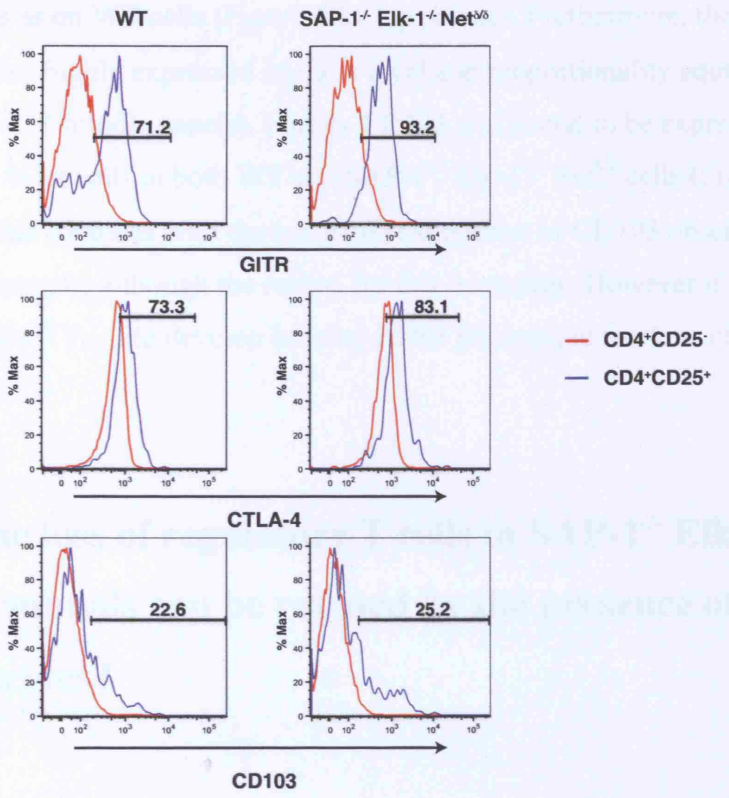


Figure 4.11. SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ CD4^{+}CD25^{+} thymocytes express regulatory markers. Expression of regulatory markers was assessed by flow cytometry analysis. GITR - top panels; CTLA-4 - middle panels; and CD103 - bottom panels.

4.9.3 SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ T_{regs} express other regulatory T cell markers.

The CD4^{+}Foxp3^{+} thymocytes present in animals reconstituted with SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ fetal liver were assessed to see if they displayed the other known markers of regulatory T cells. GITR was found to be as highly expressed on CD4^{+}CD25^{+} SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ cells as on WT cells (Figure 4.11 top panels). Furthermore, the expression of CTLA-4 was also highly expressed and at a level and proportionality equivalent to WT cells (Figure 4.11 middle panels). Finally CD103 was found to be expressed on approximately 20 % of cells in both WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ cells (Figure 4.11 bottom panels). This contrasts with the increased expression of CD103 observed in SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals, although the reason for this is unclear. However it appears that SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ T_{regs} do develop bearing all the phenotypic markers of regulatory T cells.

4.10 Can the loss of regulatory T cells in SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ animals can be rescued by the presence of WT thymocytes?

IL-2 has been shown to be important during the development of regulatory T cells although its exact role is unclear, it may be important as a survival factor rather than directly involved in development (Fontenot et al., 2005a). As a result of the severe defect in positive selection in animals reconstituted with SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ fetal livers, I considered the possibility that a lower local level of IL-2 might be responsible for T_{reg} death and reduced numbers of T_{regs}. To investigate this possibility mixed bone marrow chimera experiments were performed.

Bone marrow had previously been taken from the fetal liver SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ reconstitutions and only those with a greater than 98% contribution of donor cells were used in the subsequent mixed bone marrow reconstitutions. The SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ bone marrow was then mixed at a 1:1 ratio with BL6.SJL bone marrow allowing

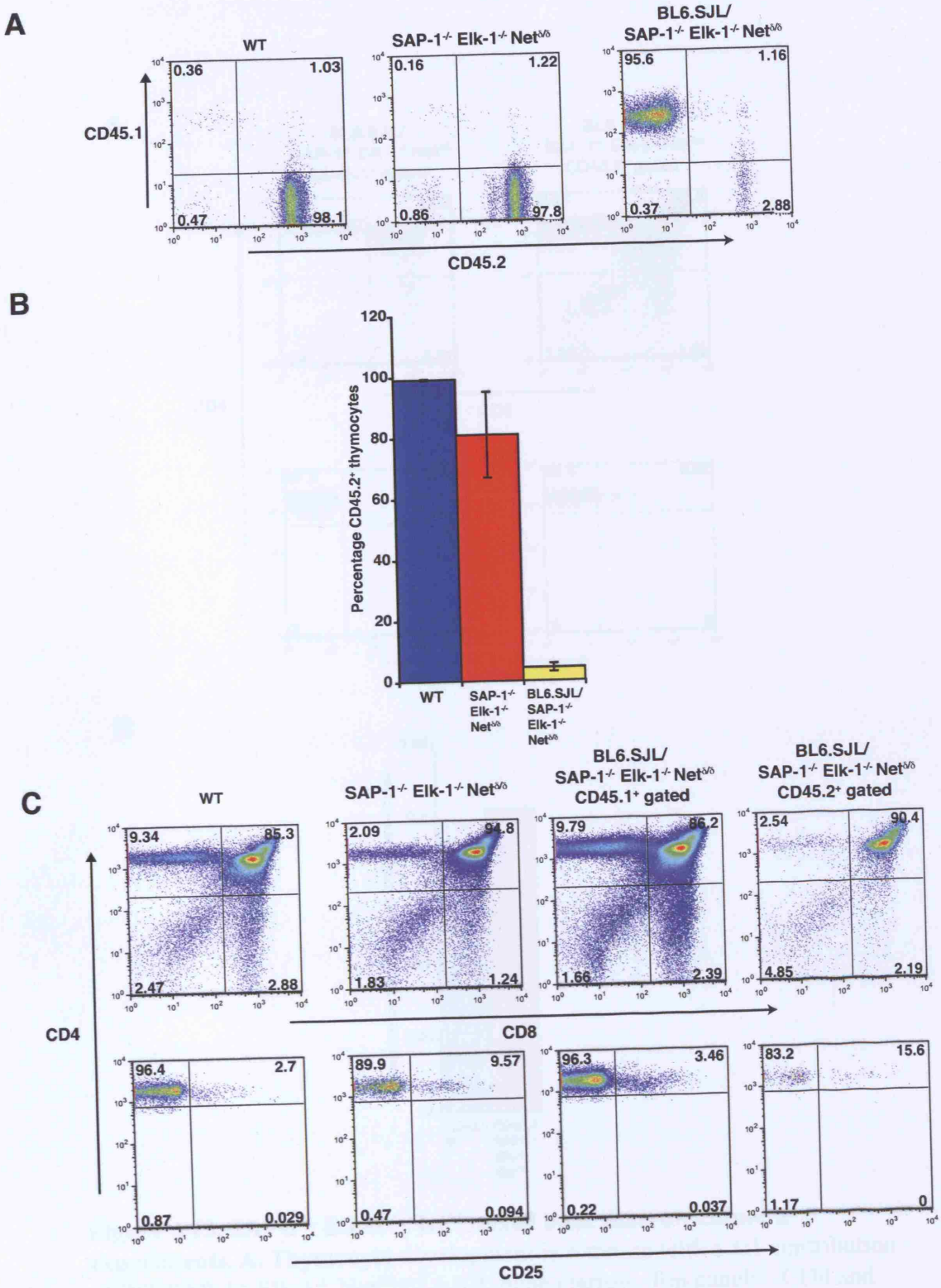


Figure 4.12. SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ mixed bone marrow chimera experiments. A. Contribution of WT, SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ and BL6.SJL bone marrow was assessed by CD45.1 and CD45. 2 expression. **B.** Average contribution of the CD45.2^{+} bone marrow across the groups (n=4). **C.** Thymoctye develoment as assessed by flow cytometry. Top row- thymic profiles as assessed by CD4 and CD8 expression. Bottom row - T_{reg} development assessed by CD25 expression on CD4^{+} gated thymocytes. Cells gated on CD45.2^{+} unless otherwise stated.

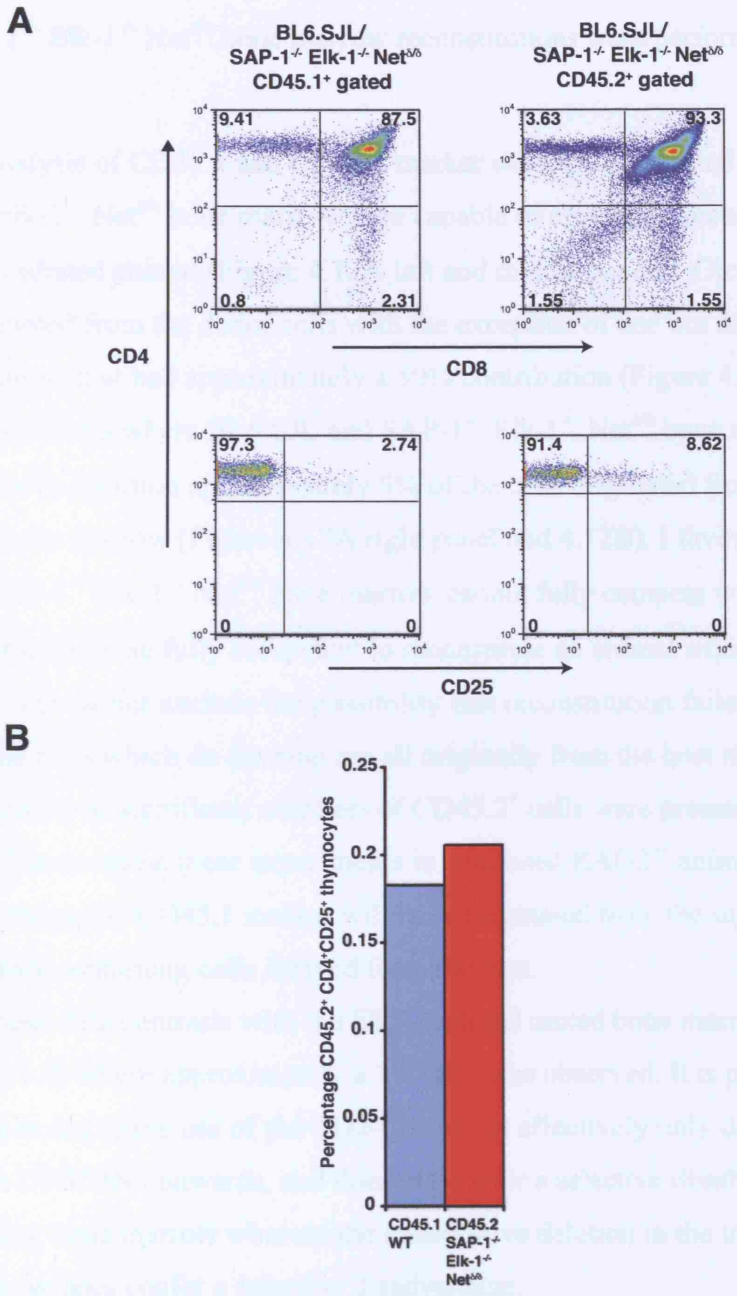


Figure 4.13. SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ mixed bone marrow chimera experiments. A. Thymocyte development in a mouse with a 1:1 contribution of WT:SAP-1$^{-/-}$ Elk-1$^{-/-}$ Net$^{\delta/\delta}$ BL6.SJL bone marrow. Top panels - CD4 and CD8 expression. Bottom panels - T_{reg} development as assessed by CD25 expression on CD4^{+} gated thymocytes. **B.** Absolute numbers of T_{regs} in this one mouse.

identification of the two populations through the CD45.1 (BL6.SJL) and CD45.2 (SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ and WT BL6) markers. This would allow a direct comparison of WT and SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ regulatory T cell development within the same animal. WT and SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow reconstitutions were performed at the same time.

Analysis of CD45.1 and CD45.2 marker expression revealed that both WT and SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow were capable of efficiently reconstituting a sub-lethally irradiated animal (Figure 4.12A left and middle panels). Greater than 97% of cells originated from the donor cells with the exception of one out of the four triple reconstitutions that had approximately a 50% contribution (Figure 4.12B). However in the reconstitutions where BL6.SJL and SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow had been mixed prior to injection approximately 5% of the cells originated from the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow (Figure 4.12A right panel and 4.12B). I favour the possibility that the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow cannot fully compete with WT bone marrow despite being fully competent to reconstitute an animal when injected alone. However, one cannot exclude the possibility that reconstitution failed in this experiment and that the cells which do develop are all originally from the host although this would appear unlikely as significant numbers of $CD45.2^+$ cells were present. One way to test this would be to repeat these experiments in irradiated $RAG2^{-/-}$ animals and thus any cells expressing the CD45.1 marker will have originated from the injected cells rather than possibly containing cells derived from the host.

These data contrasts with the SRF depleted mixed bone marrow chimeras (see section 4.11.4) where approximately a 1:1 ratio was observed. It is possible that this difference is due to the use of the CD2-Cre which effectively only deletes SRF in T cells from DN2/DN3 onwards, and does not confer a selective disadvantage to repopulating bone marrow whereas the constitutive deletion in the triple TCF deficient bone marrow does confer a selective disadvantage.

The finding that triple deficient bone marrow did not efficiently reconstituted sub-lethally irradiated animals when in competition with WT bone marrow made interpretation of the data with regards to comparing WT and triple TCF deficient T_{reg} development within the same animal very difficult. However flow cytometry analysis was performed to assess thymocyte development. Animals reconstituted with SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow showed a substantial defect in positive selection with the proportion of $CD4^+$ SP thymocytes being reduced from 9.7% ± 0.4 in WT to 2.7% ± 0.6

in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals (n=4, p=0.0002) (Figure 4.12C top row panels 1 and 2). In the mixed bone marrow chimeras analysis of $CD45.1^+$ gated cells revealed a WT profile as was expected with 8.8% ± 0.2 $CD4^+$ SP thymocytes, whilst gating on $CD45.2^+$ revealed a profile similar to the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow alone reconstitution with 3.3% ± 0.7 $CD4^+$ SP thymocytes (Figure 4.12C top row panels 3 and 4). Thus this assay recapitulated the positive selection defect observed in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ thymocytes.

T_{reg} development was assessed by the expression of CD25 in this assay due to poor $CD45.2^+$ discrimination in the Foxp3 stained samples. Analysis of CD25 expression on $CD4^+$ gated cells revealed a substantial increase in the proportion of $CD4^+CD25^+$ thymocytes in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow reconstitution from 3.3% ± 0.3 in WT to 8.9% ± 0.8 in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals (n=4, p=0.0006) (Figure 4.12C bottom row panels 1 and 2), repeating what had been seen in the fetal liver reconstitutions. In the mixed bone marrow chimeras gating on $CD45.1^+CD4^+$ showed a profile similar to that observed in WT bone marrow reconstitutions with 4.2% ± 0.2 of cells expressing CD25 however, upon gating on $CD45.2^+CD4^+$ this proportion had increased to 18.8% ± 2.8 (Figure 4.12C bottom row panels 3 and 4). The proportion observed in the CD45.2 gated cells is significantly higher than that observed in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ alone bone marrow reconstitutions (n=4, p=0.03). Therefore this suggests that the reduction in T_{reg} numbers in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ reconstitutions could be compensated for by the presence of WT thymocytes unlike the defect in positive selection.

Whilst the unequal contribution of SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ T cells in the mixed chimera experiments prevented absolute number analysis to directly compare SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ T_{reg} development with WT T_{reg} development, one of the mice from the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow experiments had resulted in a 1:1 contribution of WT thymocytes (host derived) and SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ thymocytes. Analysis of T_{reg} numbers in this one animal revealed that equivalent numbers of T_{regs} were derived from WT and SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow (Figure 4.13). Whilst this is only one mouse, these data coupled with the mixed chimera experiments suggest that the presence of WT thymocytes may be sufficient for normal T_{reg} development in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals, although further experiments would be required to confirm this.

Other possible explanations for the reduction in T_{reg} numbers in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals will be examined in the discussion (see 7.5.1).

4.11 T_{reg} development in SRF deficient animals

4.11.1 Introduction

Data presented above indicate a reduced requirement for TCF activity at least in the development of thymically derived regulatory T cells. As detailed in the introduction, recruitment of the TCFs to immediate early gene promoters is dependent on formation of a ternary complex with SRF. Deletion of SRF results in early embryonic lethality rendering the study of the immune system in SRF knockout mice impractical (Arsenian et al., 1998). An alternative approach is to conditionally delete SRF in the T cell compartment using the Cre loxP system. Two SRF conditional deletion strategies have been described (Fleige et al., 2007; Parlakian et al., 2004). A recent report using the CD4-Cre transgene to delete SRF at the DP stage of thymocyte development revealed a requirement for SRF in thymocyte development. Concurrently P. Costello in the Transcription Laboratory in collaboration with D. Degaelen has generated mice that use the CD2-Cre (Greaves et al., 1989) transgenic system designed to delete SRF early on in thymocyte development at the DN2 /DN3 stage. In these mice Cre expression deletes exon 2 which includes the MADS box domain and flanking sequences. Whilst this does result in a truncated protein being produced, deletion of the MADS box domain has been shown to result in a protein that is unable to bind DNA with no obvious dominant negative effects (Belaguli et al., 2000; Schratt et al., 2001). I have utilised these mice to examine the requirement for SRF in the generation of thymic regulatory T cells.

4.11.2 Thymocyte development in SRF depleted animals

SRF flox/flox ($SRF^{f/f}$) mice were crossed with the CD2-Cre transgene to generate a T cell specific SRF deletion ($SRF^{f/f}$ CD2-Cre^{+}). These mice are viable and exhibit no adverse outward phenotype. Efficient deletion of the SRF flox exon 2 at the

DN stage of thymocyte development has been demonstrated using real time PCR (P. Costello, R. Nicolas personal communication).

Thymocyte development was examined by flow cytometry analysis. CD4/CD8 profiles in $SRF^{f/f}$ mice are comparable to WT controls (Figure 4.14A left and middle panel). Similarly $SRF^{f/+}$ mice expressing the CD2-Cre transgene were also comparable demonstrating no adverse affect of expressing the CD2-Cre transgene on thymocyte development (data not shown). However $SRF^{f/f}$ CD2-Cre^{+} mice displayed an almost complete block in positive selection in agreement with previous reports (Fleige et al., 2007) with less than 1% $CD4^{+}$ SP thymocytes (Figure 4.14A right panel). This block is markedly more severe than that observed in the triple TCF deficient animals and may reflect an SRF-dependent / TCF-independent element of thymocyte selection.

4.11.3 T_{reg} development in SRF deficient animals

Examination of absolute numbers of $CD4^{+}$ SP thymocytes revealed an almost complete loss of cells in $SRF^{f/f}$ CD2-Cre^{+} animals (Figure 4.14B left panel). Additionally, unlike in TCF deficient animals, depletion of SRF resulted in an almost complete loss of regulatory T cells (Figure 4.14B right panel). $SRF^{f/f}$ had no affect on the numbers of either $CD4^{+}$ SP thymocytes or regulatory T cells (Figure 4.14B). The proportion of $Foxp3^{+}$ T cells in the SRF depleted animals was similar to WT (Figure 4.14C left and right panels). A similar result was also observed with $SRF^{f/f}$ (4.14C middle panel). Analysis of the residual SP thymocytes in SRF depleted animals, using real-time RT-PCR demonstrated that the majority of these cells are likely to contain at least one intact copy of the SRF gene and thus represent "escaper" cells (R. Nicolas personal communication). Furthermore the lack of T_{regs} was not as a result of the Foxp3 cells no longer expressing CD4 as when live cells were examined for Foxp3 expression, a substantial reduction was observed in the $SRF^{f/f}$ CD2-Cre^{+} animals. Thus it appears that although T_{reg} development has a low dependency on TCF activity, this process does require SRF.

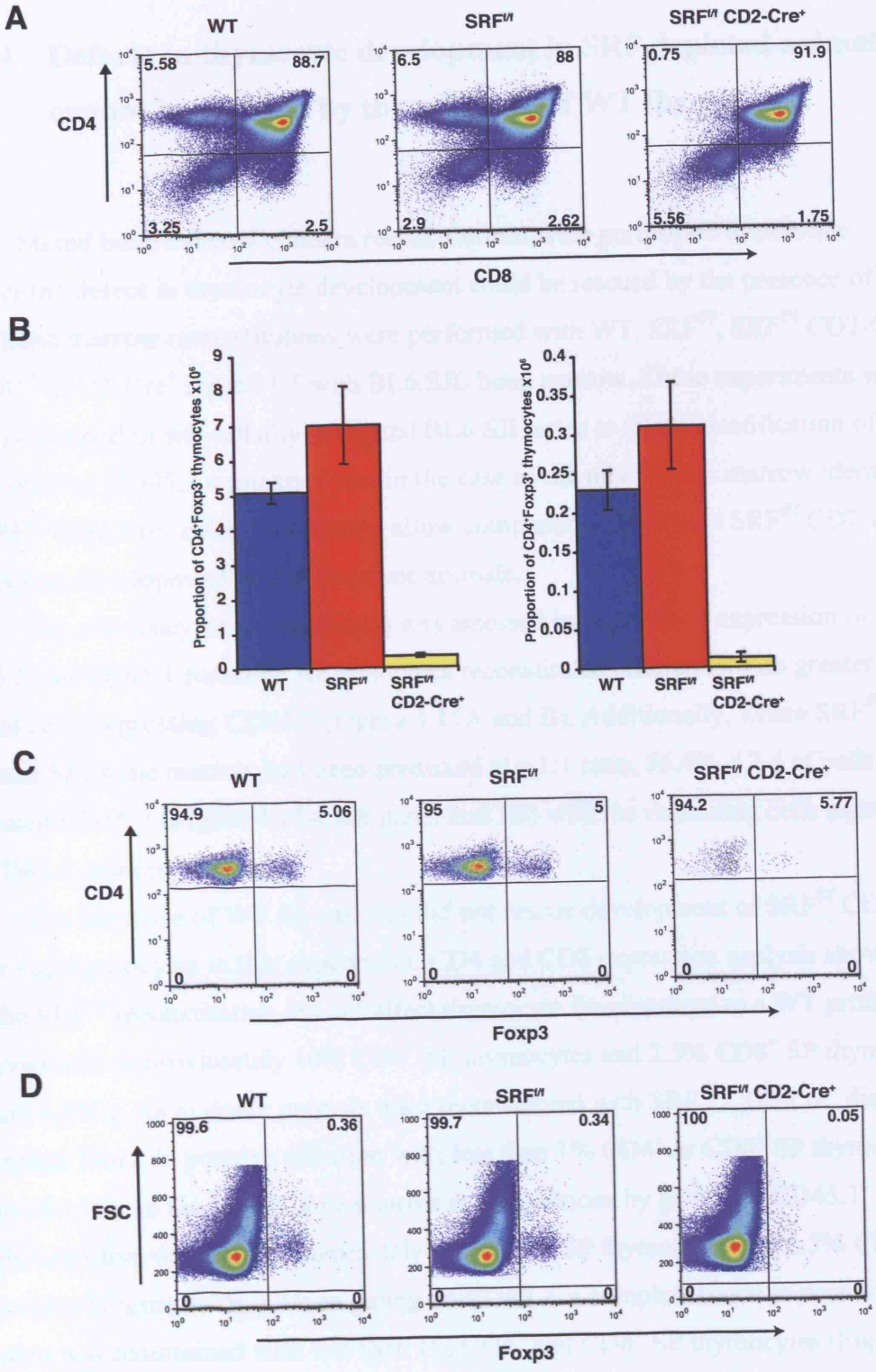


Figure 4.14. Deletion of SRF blocks thymocyte development. A. Thymocyte development was assessed by flow cytometry analysis of CD4 and CD8 expression. **B.** Absolute numbers of $CD4^+Foxp3^-$ thymocytes (left panel) and $CD4^+Foxp3^+$ thymocytes (right panel). **C.** T_{reg} development assesed by Foxp3 expression on $CD4^+$ gated thymocytes. **D.** $Foxp3^+$ cells are substantially reduced in SRF depleted animals (live gate only).

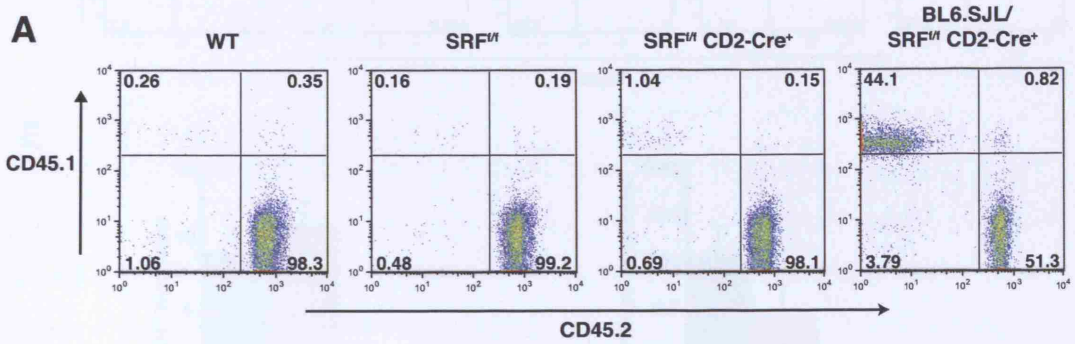
4.11.4 Defects in thymocyte development in SRF depleted animals cannot be rescued by the presence of WT thymocytes.

Mixed bone marrow chimera reconstitutions were performed to examine whether the defect in thymocyte development could be rescued by the presence of WT cells. Bone marrow reconstitutions were performed with WT, $SRF^{f/f}$, $SRF^{f/f}$ CD2-Cre^{+} and $SRF^{f/f}$ CD2-Cre^{+} mixed 1:1 with BL6.SJL bone marrow. These experiments were again performed in sub-lethally irradiated BL6.SJL mice to allow identification of donor cells by CD45.2 expression and in the case of the mixed bone marrow identify the $SRF^{f/f}$ CD2-Cre^{+} cells. This should allow comparison of WT and $SRF^{f/f}$ CD2-Cre^{+} thymocytes development within the same animals.

The efficiency of reconstitution was assessed by analysis of expression of the CD45.2 and CD45.1 markers. All genotypes reconstituted efficiently with greater than 98% of cells expressing CD45.2 (Figure 4.15A and B). Additionally, where $SRF^{f/f}$ CD2-Cre^{+} and SJL bone marrow had been premixed at a 1:1 ratio, 56.4% ± 7.4 of cells expressed CD45.2 (Figure 4.15A left panel and 2B) with the remaining cells expressing the CD45.1 marker.

The presence of WT thymocytes did not rescue development of $SRF^{f/f}$ CD2-Cre^{+} SP or T_{reg} thymocytes in this experiment. CD4 and CD8 expression analysis showed that the $SRF^{f/f}$ reconstitution did not affect thymocyte development as a WT profile was observed with approximately 10% $CD4^{+}$ SP thymocytes and 2.5% $CD8^{+}$ SP thymocytes (Figure 4.15C). As in donor animals mice reconstituted with $SRF^{f/f}$ CD2-Cre^{+} displayed a complete block in positive selection with less than 1% $CD4^{+}$ or $CD8^{+}$ SP thymocytes (Figure 4.15C). In the mixed bone marrow reconstitutions by gating on $CD45.1^{+}$ a WT profile was observed with approximately 10% $CD4^{+}$ SP thymocytes and 2.5% $CD8^{+}$ SP thymocytes (Figure 4.15C). Upon gating on $CD45.2^{+}$ a complete block in positive selection was maintained with less than 1% $CD4^{+}$ and $CD8^{+}$ SP thymocytes (Figure 4.15C).

Foxp3 expression was assessed on $CD4^{+}$ gated cells. Again $SRF^{f/f}$ did not affect thymocytes development with approximately 3% of $CD4^{+}$ SP thymocytes expressing Foxp3 that was equivalent to that seen in WT reconstitutions (Figure 4.15D). Reconstitution with $SRF^{f/f}$ CD2-Cre^{+} on the other hand resulted in a reduction in the


A
WT
SRF^f/f
SRF^f/f CD2-Cre+
BL6.SJL/
SRF^f/f CD2-Cre+
CD45.1
CD45.2
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0.35
1.06
98.3
0.16
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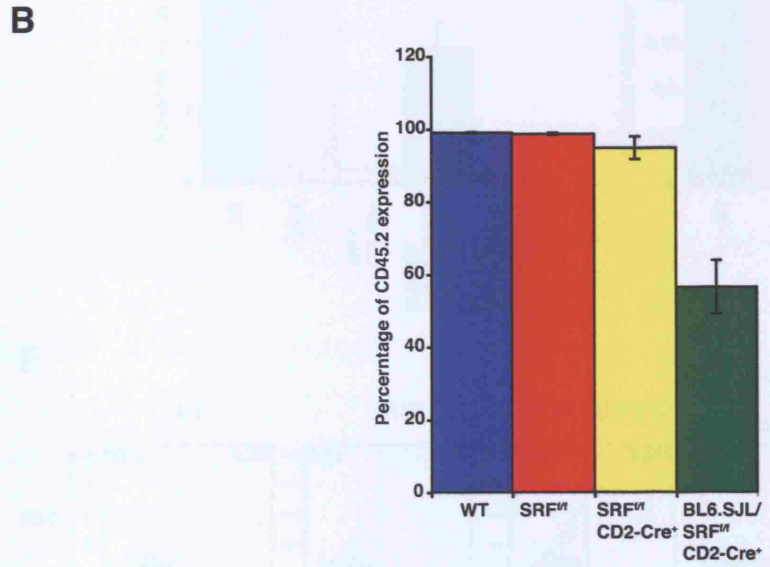
B
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100
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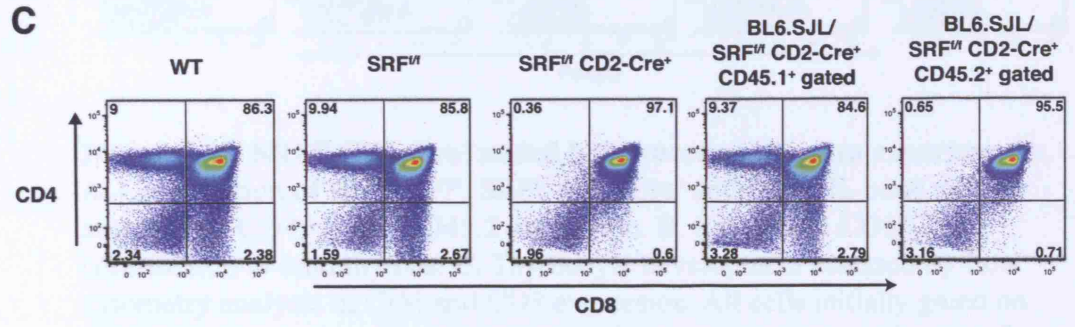
BL6.SJL/
SRF^f/f
CD2-Cre+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SRF^f/f
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SRF^f/f CD2-Cre+
CD45.2+ gated
CD4
CD8
9
86.3
2.34
2.38
9.94
85.8
1.59
2.67
0.36
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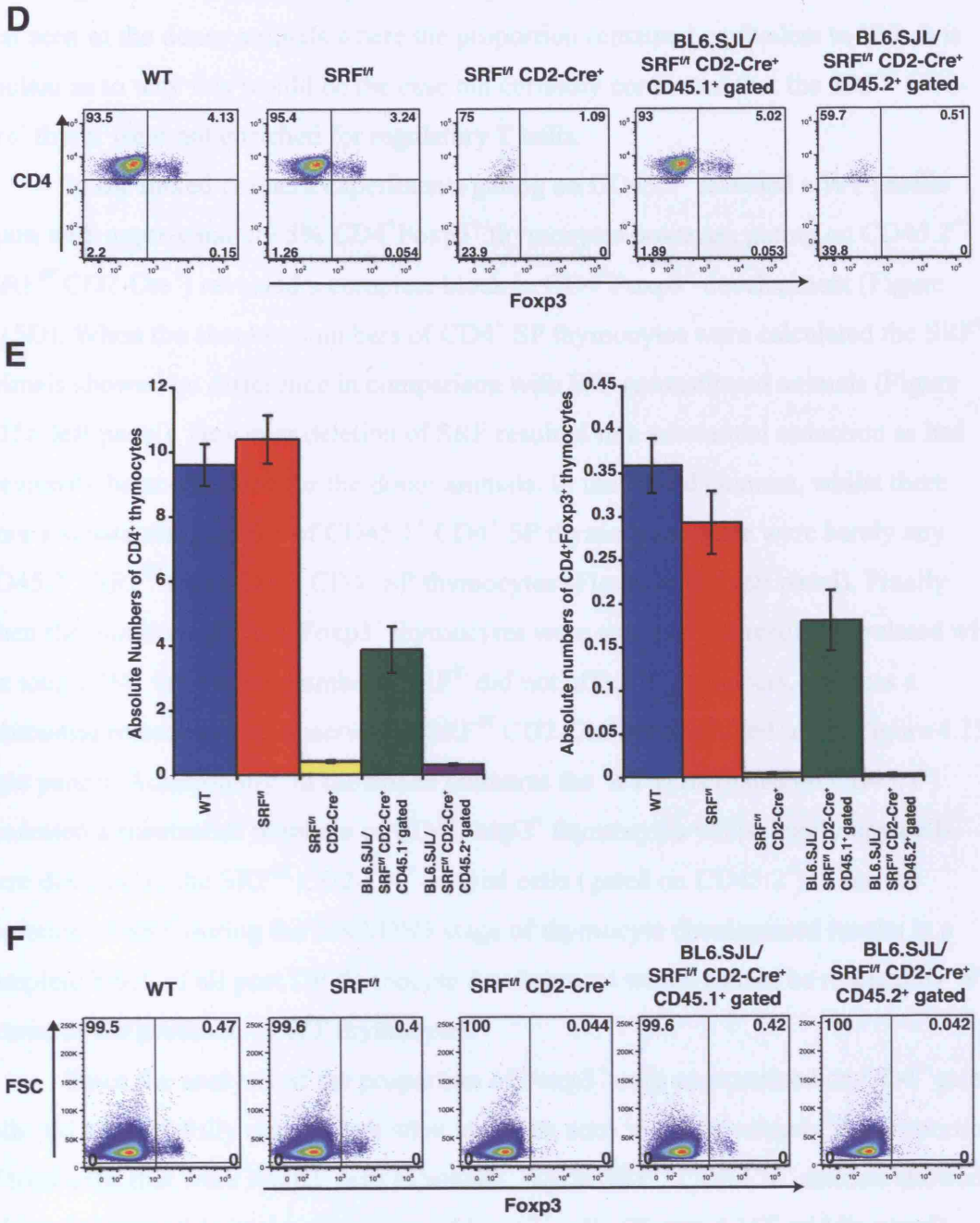


Figure 4.15. SRF$^{f/f}$ CD2-Cre^{+} mixed bone marrow chimera experiments. **A.** Contribution of WT, SRF$^{f/f}$, SRF$^{f/f}$ CD2-Cre^{+} and BL6.SJL bone marrow assessed by CD45.1 and CD45.2 expression. **B.** Percentage CD45.2^{+} contribution to total thymus. **C.** Thymocyte development assessed by flow cytometry analysis of CD4 and CD8 expression. All cells initially gated on CD45.2^{+} unless otherwise stated. **D.** T_{reg} development assessed by Foxp3 expression on CD4^{+} gated cells. All cells initially gated on CD45.2^{+} unless otherwise stated. **E.** Total numbers of CD4^{+} SP thymocytes (left panel) and CD4^{+}Foxp3^{+} thymocytes (right panel) gated on CD45.2^{+} unless otherwise stated. **F.** Total Foxp3^{+} cells are examined in all reconsitutions (n=4).

percentage of $CD4^+Foxp3^+$ to approximately 1% (Figure 4.15D). This is in contrast to that seen in the donor animals where the proportion remained equivalent to WT. It is unclear as to why this would be the case but certainly confirmed that the $SRF^{f/f}$ CD2-Cre^+ thymi were not enriched for regulatory T cells.

In the mixed chimera experiments gating on $CD45.1^+$ revealed a WT profile again with approximately 5% $CD4^+Foxp3^+$ thymocytes however, gating on $CD45.2^+$ ($SRF^{f/f}$ CD2-Cre^+) revealed a complete block in $CD4^+Foxp3^+$ development (Figure 4.15D). When the absolute numbers of $CD4^+$ SP thymocytes were calculated the $SRF^{f/f}$ animals showed no difference in comparison with WT reconstituted animals (Figure 4.15E left panel). However deletion of SRF resulted in a substantial reduction as had previously been observed for the donor animals. In the mixed chimera, whilst there were a substantial number of $CD45.1^+$ $CD4^+$ SP thymocytes, there were barely any $CD45.2^+$ ($SRF^{f/f}$ CD2-Cre^+) $CD4^+$ SP thymocytes (Figure 4.15E left panel). Finally when the numbers of $CD4^+Foxp3^+$ thymocytes were assessed the results correlated with the total $CD4^+$ thymocyte numbers. $SRF^{f/-}$ did not affect T_{reg} numbers, whereas a substantial reduction was observed in $SRF^{f/f}$ CD2-Cre^+ reconstituted mice (Figure 4.15E right panel). Additionally, in the mixed chimeras the WT cells (gated on $CD45.1^+$) generated a substantial numbers of $CD4^+Foxp3^+$ thymocytes whilst barely any cells were detected in the $SRF^{f/f}$ CD2-Cre^+ derived cells (gated on $CD45.2^+$). Thus the depletion of SRF during the DN2/DN3 stage of thymocyte development results in a complete block of all post DP thymocyte development which cannot be rescued by WT stroma or the presence of WT thymocytes.

Since the analysis of the proportion of $Foxp3^+$ cells as examined on $CD4^+$ gated cells did not faithfully recapitulate what had been seen in donor animals, the proportion of total cells that were $Foxp3^+$ was examined. Again $SRF^{f/f}$ CD2-Cre^+ animals showed a substantial reduction in the proportion of $Foxp3^+$ cells (Figure 4.15F middle panel). This was also observed in the mixed chimera experiments when $CD45.2^+$ cells were examined (Figure 4.15F far right panel). Thus SRF is required for T_{reg} development and this cannot be compensated for by the presence of WT thymocytes.

These results raise interesting questions about the difference between the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals and $SRF^{f/f}$ CD2-Cre^+ animals which will be expanded on in the discussion (see 7.5.2).

4.12 Summary

This chapter addresses whether the TCFs Elk-1 and Net had a specific role in T_{reg} development. Furthermore the possibility that T_{reg} development simply has a lower requirement for TCF signalling compared to the positive selection of conventional T cells was addressed. Analysis of Elk-$1^{-/-}$ or $Net^{\delta/\delta}$ mice demonstrated that T_{reg} development was unaffected in these animals. Furthermore loss of either Elk-1 or Net activity on a SAP-$1^{-/-}$ background did not result in a reduced number of T_{regs} in these animals, although this did demonstrate that SAP-1 and Elk-1 act redundantly in positive selection. T_{reg} development was also assessed in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ cells by making use of fetal liver reconstitutions. The proportion of $Foxp3^{+}$ thymocytes in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ reconstitutions was similar to that observed for the SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals demonstrating that T_{regs} do develop in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals. Analysis of absolute numbers was complicated by the fact that these experiments do not faithfully replicate the increased thymus size observed in SAP-$1^{-/-}$ Elk-$1^{-/-}$ donor animals. There appeared to be a slight reduction in the numbers of T_{regs} which developed in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals although mixed bone marrow chimera experiments suggest that this could might be rescued by the presence of WT thymocytes. These data suggest that at a minimum, T_{reg} development has a lower requirement for TCF activity than positive selection.

Examination of the conditional deletion of the TCF transcription partner SRF revealed a complete block in positive selection as had previously been described. Interestingly and in contrast to the TCF deficient animals, there was also a complete block in regulatory T cell development, although why this is the case is unclear. These data indicate that there is another SRF-dependent function that is required for all thymocyte development and that there is an SRF-dependent TCF-independent process within positive selection

5 Results – Regulatory T cell function in TCF deficient animals

5.1 Abstract

Regulatory T cells are important in the prevention of autoimmunity as well as regulating immune responses. In *in vitro* studies and *in vivo* models regulatory T cells have been shown to suppress effector T cell proliferation and function. The ability to suppress effector cells requires Foxp3 expression and activation through the TCR. Here I demonstrate that TCR activation of T_{regs} induces ERK signalling and Egr-1 up-regulation but this is of a lower magnitude than conventional $CD4^+$ T cells. Furthermore TCF deficient T_{regs} are fully functional *in vitro* and most likely *in vivo*.

5.2 Introduction

The importance of regulatory T cells has been demonstrated in many models of autoimmunity. Depletion of $CD4^+CD25^+$ T cells has been shown to lead to autoimmunity whilst addition of these same cells can suppress and cure autoimmunity (Sakaguchi et al., 1995; Itoh et al., 1999; Mottet et al., 2003). The $CD4^+CD25^+$ T cell population has also been shown to suppress proliferation of effector cells *in vitro* (Sakaguchi et al., 1995; Itoh et al., 1999; Takahashi et al., 1998), thus the $CD4^+CD25^+$ T cell population has been used to identify regulatory T cells for use in functional studies. These cells are naturally anergic *in vitro* and have been reported to have distinct responses to TCR activation when compared to conventional $CD4^+$ T cells (Hickman et al., 2006). It has been suggest that the impaired ERK activation in T_{reg} cells may in part explain the anergic phenotype of these cells *in vitro*. However activation through the TCR is required for T_{reg} function (Thornton and Shevach, 1998; Takahashi et al., 1998; Piccirillo et al., 2002).

TCF deficient T_{regs} were phenotypically indistinct from WT T_{regs} as assessed by the expression of regulatory markers however; it remained possible that the TCFs could

have a role in regulatory T cell function. The TCFs are important mediators of TCR signalling and are required for efficient proliferation, at least in conventional T cells. Thus the responses to TCR activation were examined in WT and SAP-$1^{-/-}$ T_{regs}. Furthermore SAP-$1^{-/-}$ and SAP-$1^{-/-}$ Elk-$1^{-/-}$ T_{regs} were assessed for regulatory function using both *in vitro* and *in vivo* assays.

5.3 ERK signalling in regulatory T cells

The suppressive effects of T_{regs} *in vitro* require activation of the TCR (Thornton and Shevach, 1998; Takahashi et al., 1998; Piccirillo et al., 2002). As a prelude to functional studies the responses of TCF deficient T_{regs} to TCR activation were examined. The differential requirement for TCF signalling in the development of conventional $CD4^+$ T cells and $Foxp3^+$ regulatory T cells suggested that there may be differences in the signalling pathways downstream of TCR ligation. Activation of the TCR was shown to activate the Ras-ERK signalling pathway (Downward et al., 1990; Delgado et al., 2000). The loss of SAP-1 may affect TCR signalling in two ways, either at the level of signal generation or at signal execution, either of which could affect T_{reg} function. Therefore I examined whether ERK signalling could be activated through the TCR in regulatory T cells.

Analysis of ERK activation on a cell-by-cell basis in thymic regulatory T cells was performed by intracellular staining. Thymocytes were isolated and rested prior to activation. Activation of the TCR by pre-coating with αCD3 and then crosslinking with goat anti-hamster antibody results in the phosphorylation of the ERK activation-loop as detected by antibodies specific for this phosphorylation. Cells were crosslinked for various times before being fixed in 4% paraformaldehyde to terminate the reaction. The cells were then stained with CD4, CD25, CD8 and p-ERK. Cells were gated on $CD4^+CD25^-$ and $CD4^+CD25^+$ populations for flow cytometry analysis. In both $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells maximal induction of phosphorylated ERK appeared to be between 1 and 2 min post-crosslinking (Figure 5.1A). Previously it has been shown that the maximal induction occurred at 2 min post-crosslinking in DP thymocytes (Costello et al., 2004). Induction of p-ERK in $CD4^+CD25^+$ T cells was readily detected, but appeared less efficient than that observed in conventional $CD4^+CD25^-$ thymocytes. It was possible to block this induction of p-ERK

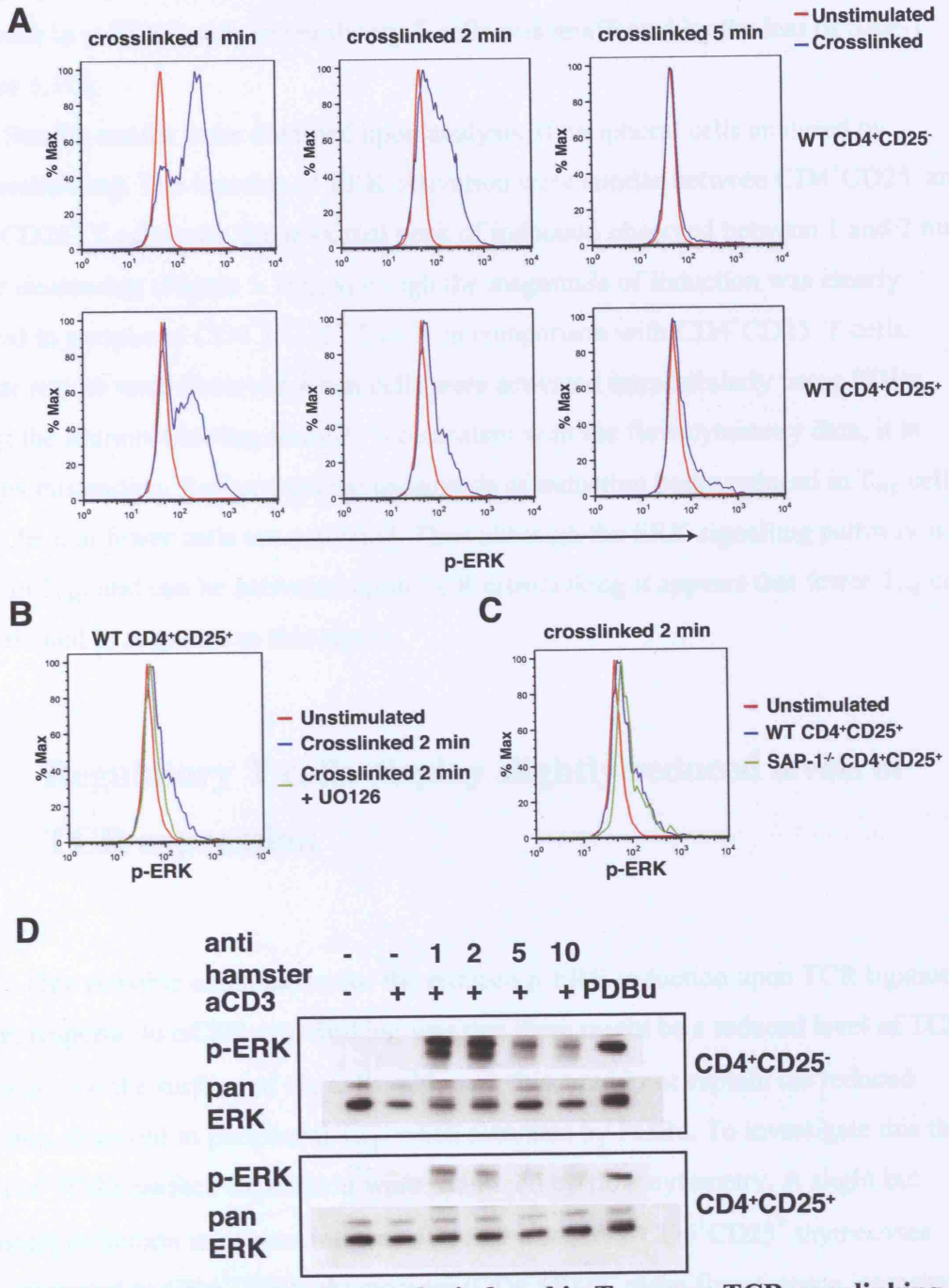


Figure 5.1. ERK activation can be induced in T_{regs} upon TCR crosslinking. **A-C.** Total thymocytes were activated by αCD3 and crosslinking with goat anti-hamster for 1, 2 and 5 min. ERK phosphorylation was assessed by intracellular staining. **A.** Cells were gated on either $CD4^+CD25^-$ or $CD4^+CD25^+$ and kinetics of ERK actiavtion determined. **B.** Thymocytes where stated were pre-treated with UO126 and activation of ERK assessed in $CD4^+CD25^+$ T cells. **C.** WT and SAP-$1^{-/-}$ $CD4^+CD25^+$ T cells induce ERK phosphorylation to the same extent. Cells were activated for 2 min. **D.** Lymph node cells sorted into $CD4^+CD25^-$ and $CD4^+$ $CD25^+$ populations and either activated by αCD3 and crosslinking for various times or were activated by PDBu for 10 min. ERK and phosphorylated ERK protein was determined by western blot analysis.

in T_{regs} by the addition of the MEK inhibitor UO126 (Figure 5.1B). Furthermore this induction in p-ERK levels in regulatory T cells was unaffected by the loss of SAP-1 (Figure 5.1C).

Similar results were obtained upon analysis of peripheral cells analysed by immunoblotting. The kinetics of ERK activation were similar between $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells with the maximal peak of induction observed between 1 and 2 min before decreasing (Figure 5.1D), although the magnitude of induction was clearly reduced in peripheral $CD4^+CD25^+$ T cells in comparison with $CD4^+CD25^-$ T cells. Similar results were observed when cells were activated intracellularly using PDBu. Whilst the immunoblotting analysis is consistent with the flow cytometry data, it is perhaps misleading. Rather than the magnitude of induction being reduced in T_{reg} cells, it may be that fewer cells are activated. Thus although the ERK signalling pathway is intact in T_{regs} and can be activated upon TCR crosslinking it appears that fewer T_{reg} cells are activated in response to this signal.

5.4 Regulatory T cells display slightly reduced levels of TCR expression

One possible explanation for the reduced p-ERK induction upon TCR ligation in T_{regs} in response to αCD3 crosslinking was that there might be a reduced level of TCR expression on the surface of the cells, although this would not explain the reduced induction observed in peripheral T_{regs} when activated by PDBu. To investigate this the levels of TCRβ surface expression were examined by flow cytometry. A slight but consistent reduction in expression was detected when WT $CD4^+CD25^+$ thymocytes were compared to $CD4^+CD25^-$ thymocytes ($CD4^+CD25^-$ mean fluorescence intensity (MFI) 3781 ± 98.43; $CD4^+CD25^+$ MFI 2844 ± 92.22; p= 0.002, n=3) (Figure 5.2A left panel). This was also observed when peripheral $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were compared ($CD4^+CD25^-$ MFI 2952 ± 162.7; $CD4^+CD25^+$ MFI 2475 ± 66.98; p= 0.01, n=4) (Figure 5.2A right panel). The loss of SAP-1 did not have an effect on TCR levels in $CD4^+CD25^-$ or $CD4^+CD25^+$ thymocytes (Figure 5.2B).

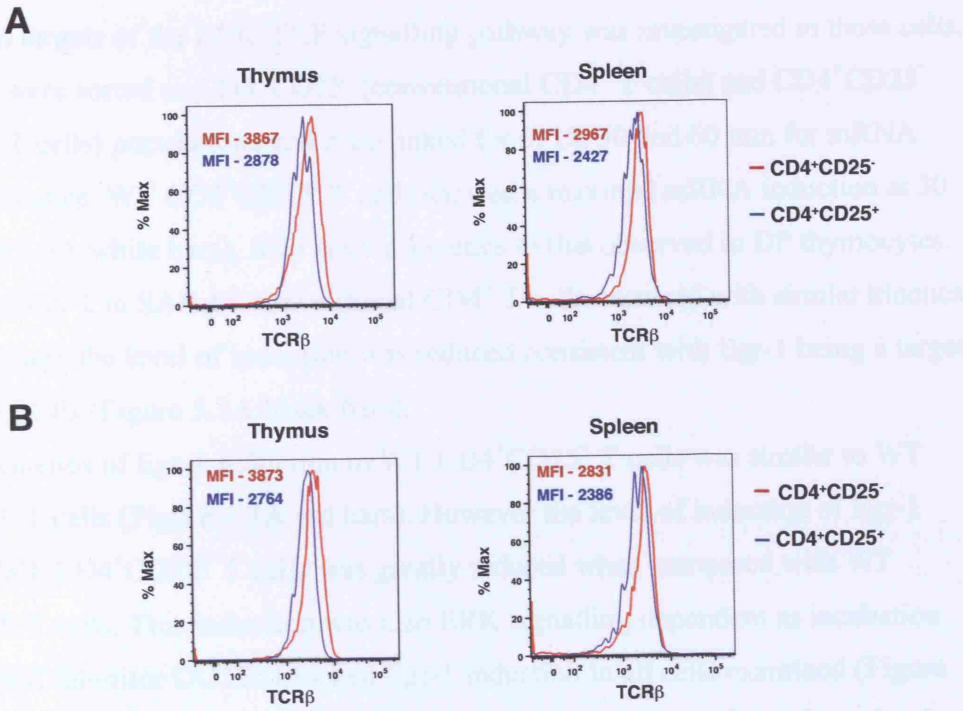


Figure 5.2. Reduced expression of TCRβ on T_{regs}. A. TCRβ expression was measured on WT $CD4^+CD25^-$ (red line) and $CD4^+CD25^+$ (blue line) cells isolated from thymus and spleen. Mean Fluoresence Intensity (MFI) is shown inset. **B.** TCRβ expression was measured on SAP-$1^{-/-}$ $CD4^+CD25^-$ (red line) and SAP-$1^{-/-}$ $CD4^+CD25^+$ (blue line) cells isolated from thymus and spleen. MFI is shown inset.

5.5 TCF target genes can be induced in T_{regs}

In addition to induction of ERK phosphorylation, TCR crosslinking of DP thymocytes results in the induction of TCF target genes, in particular Egr-1. SAP-$1^{-/-}$ DP thymocytes showed a reduced induction of Egr-1 mRNA and protein levels (Costello et al., 2004). Since the ERK pathway could be activated in T_{regs}, the downstream targets of the ERK-TCF signalling pathway was investigated in these cells.

Cells were sorted as $CD4^+CD25^-$ (conventional $CD4^+$ T cells) and $CD4^+CD25^+$ (regulatory T cells) populations and crosslinked for 0, 15, 30 and 60 min for mRNA expression studies. WT $CD4^+CD25^-$ T cells showed a maximal mRNA induction at 30 min (Figure 5.3A white bars), with similar kinetics to that observed in DP thymocytes. Induction of Egr-1 in SAP-$1^{-/-}$ conventional $CD4^+$ T cells occurred with similar kinetics to WT, although the level of induction was reduced consistent with Egr-1 being a target gene of the TCFs (Figure 5.3A black bars).

The kinetics of Egr-1 induction in WT $CD4^+CD25^+$ T cells was similar to WT $CD4^+CD25^-$ T cells (Figure 5.3A red bars). However the level of induction of Egr-1 mRNA in WT $CD4^+CD25^+$ T cells was greatly reduced when compared with WT $CD4^+CD25^-$ T cells. This induction was also ERK signalling dependent as incubation with the MEK inhibitor UO126 blocked Egr-1 induction in all cells examined (Figure 5.3A). When Egr-1 induction was examined in SAP-$1^{-/-}$ $CD4^+CD25^+$ T cells, induction of Egr-1 was barely detectable (Figure 5.3A blue bars). This is consistent with the view that Egr-1 induction is lower in T_{regs} and removal of SAP-1 reduces this further.

In order to examine whether the reduced Egr-1 mRNA induction in T_{regs} correlated with reduced induction of protein expression, intracellular staining was performed as previously described in Costello et al 2004. The Egr-1 antibody used has previously demonstrated to identify a single band with the same molecular weight as determined for Egr-1 and shown to be inducible in response to TCR crosslinking. Furthermore this antibody is also capable of recognising transfected Egr-1 expressed in phoenix cells and analysed by immunoprecipitation (Figure 5.3B). In DP thymocytes and conventional $CD4^+CD25^-$ T cells, TCR activation resulted in a significant increase in Egr-1 protein levels after 4 hours (Costello et al., 2004 and Figure 5.3C left panel). This increase is dependent on both SAP-1 and ERK signalling as shown by the reduced

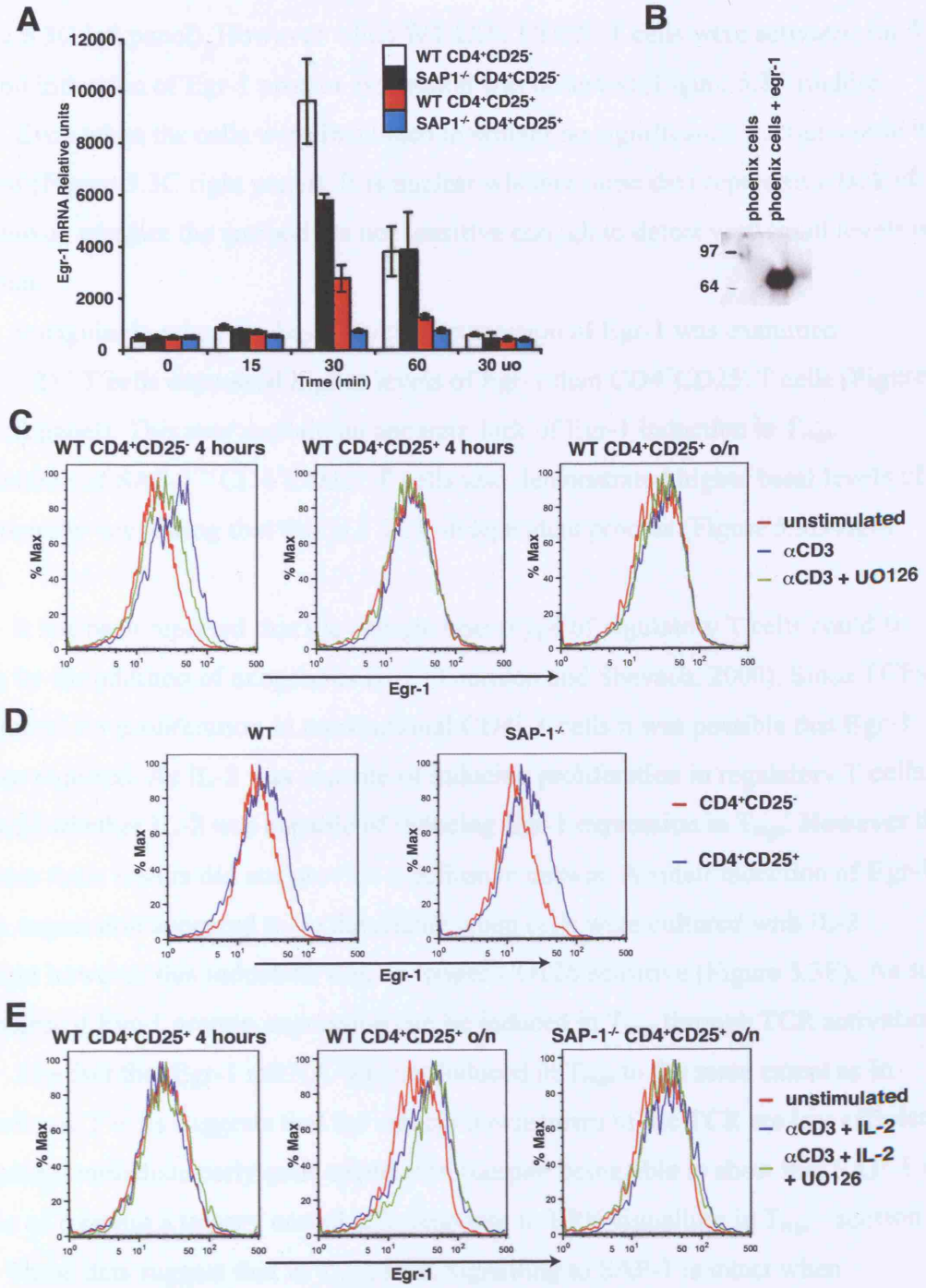


Figure 5.3. Inefficient Egr-1 induction in SAP-1$^{-/-}$ T_{regs}. A. Egr-1 mRNA induction was examined by real-time RT-PCR, expression was normalised to HPRT. Cells were sorted into CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ populations and activated with αCD3. Where stated cells were pre-treated with UO126. **B.** I.P. performed on phoenix cells transfected with retrovirus containing Egr-1. IP with α-Flag then immunblot with α-Egr-1. **C-E.** Egr-1 protein induction was followed by intracellular staining of sorted CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ T cells. **C.** WT cells were activated with αCD3 for 4 hours or overnight **D.** Unstimulated levels of Egr-1 were analysed at 4 hours in WT and SAP-1$^{-/-}$ CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ T cells. **E.** WT and SAP-1$^{-/-}$ CD4$^+$CD25$^+$ T cells were activated with αCD3, APCs and IL-2 for 4 hours or overnight.

induction in the SAP-$1^{-/-}$ T cells and cells treated with the MEK inhibitor UO126 (Figure 5.3C left panel). However when WT $CD4^+CD25^+$ T cells were activated for 4 hours no induction of Egr-1 protein expression was observed (Figure 5.3C middle panel). Even when the cells were incubated overnight no significant induction could be detected (Figure 5.3C right panel). It is unclear whether these data represent a lack of induction or whether the antibody is not sensitive enough to detect very small levels of induction.

Intriguingly when the basal level of expression of Egr-1 was examined $CD4^+CD25^+$ T cells expressed higher levels of Egr-1 than $CD4^+CD25^-$ T cells (Figure 5.3D left panel). This may explain an apparent lack of Egr-1 induction in T_{regs}. Examination of SAP-$1^{-/-}$ $CD4^+CD25^+$ T cells also demonstrated higher basal levels of Egr-1 thereby suggesting that this is a TCF-independent process (Figure 5.3D right panel).

It has been reported that the anergic phenotype of regulatory T cells could be broken by the addition of exogenous IL-2 (Thornton and Shevach, 2000). Since TCFs are required for proliferation in conventional $CD4^+$ T cells it was possible that Egr-1 was also required. As IL-2 was capable of inducing proliferation in regulatory T cells, I addressed whether IL-2 was capable of inducing Egr-1 expression in T_{regs}. However the data from these results did not provide a definitive answer. A small induction of Egr-1 protein expression appeared to be detectable when cells were cultured with IL-2 overnight however this induction was no longer UO126 sensitive (Figure 5.3E). As such it is unclear if Egr-1 protein expression can be induced in T_{regs} through TCR activation.

The fact that Egr-1 mRNA was not induced in T_{regs} to the same extent as in conventional T cells suggests that the signals downstream of the TCR are less efficient at inducing immediate early gene expression (despite being able to show that SAP-1 is capable of forming a ternary complex in response to ERK signalling in T_{regs} - section 3.10). These data suggest that in T_{regs}, ERK signalling to SAP-1 is intact when compared with conventional $CD4^+$ T cells however the activation of immediate early genes downstream of ERK and SAP-1 is reduced in T_{regs}. This finding may in part underpin the anergic phenotype described for T_{regs} *in vitro* in that the lack of induction of immediate early genes may result in a lack of proliferative capabilities.

5.6 Are TCF deficient T_{regs} functional *in vitro*?

5.6.1 SAP-$1^{-/-}$ deficient T_{regs} are functional *in vitro*

The classical test of T_{reg} function *in vitro* is the ability to suppress proliferation of effector cells. $CD4^+CD25^-$ T cells (effector cells) were activated with soluble αCD3 and APCs (T cell depleted irradiated splenocytes) for 72 hours. The proliferation of these cells is determined either by ^3H labelled thymidine incorporation or by CFSE dilution. CFSE (Carboxyfluoroscein succinimidyl ester) is cleaved by non-specific esterases after entering the cell and remains in the cytoplasm of the cell for days to months afterwards. If cells are dividing then the amount of CFSE present in each daughter cell after one division is half that of the parent cell thus proliferation can be followed by the dilution of CFSE. Addition of regulatory T cells to these cultures inhibits proliferation of the effector cells with maximal suppression occurring at a 1 T_{reg}:1 Effector cell ratio (Thornton and Shevach., 1998; Takahashi et al., 1998; Piccirillo et al., 2002). Suppression is independent of suppressive cytokines and is contact dependent although the mechanism remains unclear.

$CD4^+CD25^-$ effector cells and $CD4^+CD25^+$ regulatory T cells were isolated by cell sorting from lymph nodes. Sorted populations were then mixed at ratios ranging from 1 T_{reg}:1 Effector cell to a 1 T_{reg}:8 Effector cells ratio. Initial experiments used ^3H labelled thymidine incorporation to assess the level of proliferation. When WT $CD4^+CD25^-$ T cells alone were activated substantial proliferation could be detected (Figure 5.4A). In contrast when WT or SAP-$1^{-/-}$ $CD4^+CD25^+$ T cells were incubated alone very little proliferation could be detected (Figure 5.4A) consistent with previous reports (Thornton and Shevach, 1998; Takahashi et al., 1998; Piccirillo et al., 2002). Thus the SAP-$1^{-/-}$ $CD4^+CD25^+$ T cells not only expressed the regulatory T cell markers they also displayed the anergic *in vitro* phenotype of regulatory T cells.

Upon addition of increasing amounts of WT regulatory T cells the level of proliferation of $CD4^+CD25^-$ T cells decreased in a dose dependent manner (Figure 5.4A) with proliferation being suppressed by approximately 80% at a 1:1 ratio. This reduction is unlikely to reflect nutrient limitation under the culture conditions since

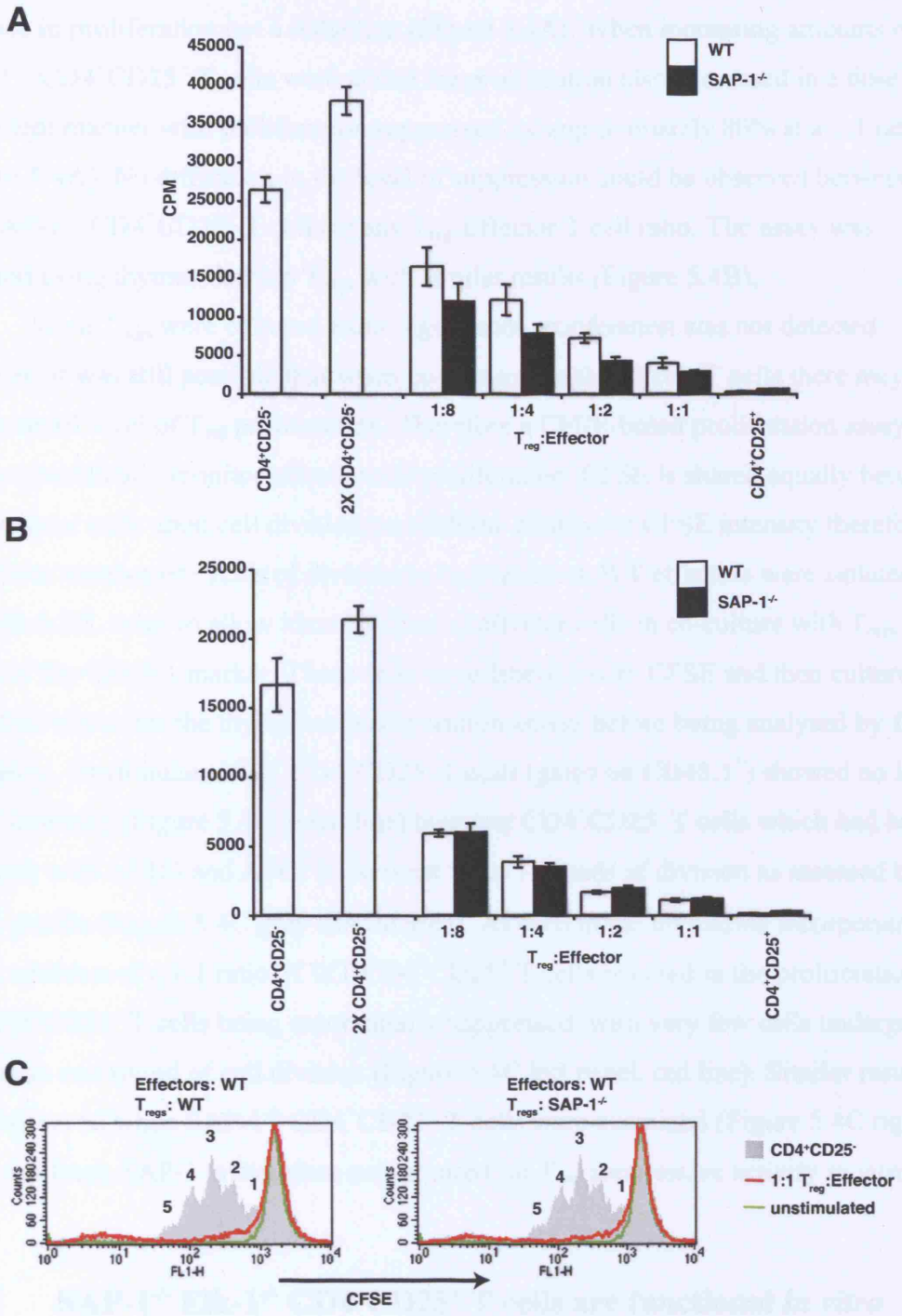


Figure 5.4. SAP-1$^{-/-}$ T_{regs} are functional *in vitro*. *In vitro* suppression assays were performed. Cells were sorted into $CD4^+CD25^-$ and $CD4^+CD25^+$ populations, then activated with αCD3 and APCs for 72 hours. **A.** and **B.** Proliferation assessed by ^3H labelled thymidine incorporation. $CD4^+CD25^+$ T cells were added in increasing amounts. **A.** Cells isolated from lymph nodes **B.** Cells isolated from thymi **C.** Cells sorted from lymph nodes and proliferation assessed by CFSE dilution. $CD4^+CD25^+$ T cells were added at a 1:1 ratio of T_{reg}:Effector

addition of $CD4^+CD25^-$ T cells to the equivalent cell number of a 1:1 ratio led to an increase in proliferation not a reduction (Figure 5.4A). When increasing amounts of SAP-$1^{-/-}$ $CD4^+CD25^+$ T cells were added the proliferation also decreased in a dose dependent manner with proliferation suppressed by approximately 80% at a 1:1 ratio (Figure 5.4A). No difference in the level of suppression could be observed between WT and SAP-$1^{-/-}$ $CD4^+CD25^+$ T cells at any T_{reg}:Effector T cell ratio. The assay was repeated using thymus derived T_{regs} with similar results (Figure 5.4B).

When T_{regs} were cultured alone significant proliferation was not detected however, it was still possible that when co-cultured with effector T cells there may have been a small level of T_{reg} proliferation. Therefore a CFSE-based proliferation assay was used to specifically monitor effector cell proliferation. CFSE is shared equally between the daughter cells upon cell division; as such the dilution of CFSE intensity therefore allows the number of cycles of division to be evaluated. WT effectors were isolated from BL6.SJL mice to allow identification of effector cells in co-culture with T_{regs} by virtue of the CD45.1 marker. These cells were labelled with CFSE and then cultured as described above for the thymidine incorporation assays before being analysed by flow cytometry. Unstimulated WT $CD4^+CD25^-$ T cells (gated on $CD45.1^+$) showed no loss of CFSE intensity (Figure 5.4C green line) however $CD4^+CD25^-$ T cells which had been activated with αCD3 and APCs underwent up to 5 rounds of division as assessed by the CFSE profile (Figure 5.4C grey shaded area). As seen in the thymidine incorporation assay, addition of a 1:1 ratio of WT $CD4^+CD25^+$ T cells resulted in the proliferation of the $CD4^+CD25^-$ T cells being substantially suppressed, with very few cells undergoing more than one round of cell division (Figure 5.4C left panel, red line). Similar results were observed when SAP-$1^{-/-}$ $CD4^+CD25^+$ T cells were examined (Figure 5.4C right panel red line). SAP-1 is therefore not required for T_{reg} suppressive activity *in vitro*.

5.6.2 SAP-$1^{-/-}$ Elk-$1^{-/-}$ $CD4^+CD25^+$ T cells are functional *in vitro*

To address the possibility of threshold or TCF specific effects in T_{reg} function, SAP-$1^{-/-}$ Elk-$1^{-/-}$ T_{regs} were also assessed for functionality in the *in vitro* suppression assays. These assays were performed with cells isolated from the periphery due to the difficulty in getting large numbers of these mice due to fertility issues (P.C and R.T

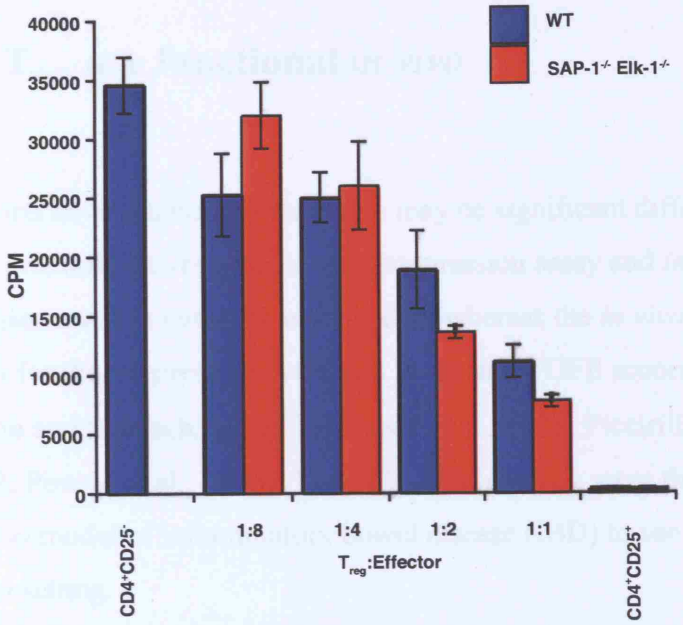


Figure 5.5. SAP-1$^{-/-}$ Elk-1$^{-/-}$ T_{regs} are functional *in vitro*. *In vitro* suppression assays were performed. Cells were sorted from lymph nodes into $CD4^+CD25^-$ and $CD4^+CD25^+$ populations, then activated with αCD3 and APCs for 72 hours. Proliferation was assessed by ^3H labelled thymidine incoproation. $CD4^+CD25^+$ T cells were added in increasing amounts.

unpublished observations). As with WT and SAP-1$^{-/-}$ CD4$^+$CD25$^+$ T cells, SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4$^+$CD25$^+$ T cells did not proliferate when incubated with αCD3 and APCs for 72 hours (Figure 5.5). Furthermore addition of these cells to WT CD4$^+$CD25$^-$ T cell cultures suppressed proliferation in a dose dependent manner (Figure 5.5). At a 1:1 ratio of SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4$^+$CD25$^+$ T cells to WT effector cells approximately 80% suppression was observed and at all ratios examined no significant difference could be observed between the addition of WT and SAP-1$^{-/-}$ Elk-1$^{-/-}$ CD4$^+$CD25$^+$ T cells. Thus the function of regulatory T cells appears to be independent of both SAP-1 and Elk-1.

5.7 SAP-1$^{-/-}$ T_{regs} are functional *in vivo*

Previous reports have established that there may be significant differences in the requirements for suppression between the *in vitro* suppression assay and *in vivo* models. The *in vitro* suppression assay is cytokine independent whereas the *in vivo* models have shown a requirement for the suppressive cytokines IL-10 and TGFβ according to the model used (Thornton and Shevach, 1998; Takahashi et al., 1998; Piccirillo et al., 2002; Asseman et al., 1999; Powrie et al., 1996). The TCF deficient T_{regs} were therefore examined in an *in vivo* model of inflammatory bowel disease (IBD) to see whether they function in an *in vivo* setting.

In the *in vivo* model of IBD used here, symptoms of colitis are induced by the transfer of naïve effector T cells into immunodeficient mice and the function of T_{reg} cells is then assessed by their ability to suppress or cure the development of colitis upon co-transfer or subsequent transfer into the recipient mice (Mottet et al., 2003; Powrie et al., 1994; Powrie et al., 1993). Both IL-10 and TGFβ have been shown to be important in this suppression (Asseman et al., 1999; Powrie et al., 1996).

Depending on the strain of mice used, significant weight loss can be detected at about 6 weeks post intraperitoneal (ip) injection. Other clinical symptoms also become apparent at this time such as loose stools, T cell infiltrates in the colon, loss of goblet cells and elongation of crypt length. All of these factors are then assessed to give a score from 0 to 4 as to the severity of the disease with 0-1 being no disease, 2 being mild and 3 and 4 being severe disease.

Cells were isolated from spleen and naïve effectors were sorted as CD45RB^{hi}CD4$^+$CD25$^-$ whilst T_{regs} were sorted as CD45RB^{lo}CD4$^+$CD25$^+$. Weight loss

was monitored as the experiment progressed and at 5 weeks severe weight loss was observed in both WT effector alone and SAP-$1^{-/-}$ effector alone groups (Figure 5.6A blue and red lines respectively). Once mice had lost 20% of their weight, they were sacrificed as directed by CRUK ethics guidelines. The groups in which either WT or SAP-$1^{-/-}$ T_{regs} had been co-injected at a 1 T_{reg}:2 Effector T cell ratio, displayed no weight loss, suggesting that they may be suppressing that aspect of the disease (Figure 6A yellow and green lines respectively).

The weight loss observed in the WT effectors alone and the SAP-$1^{-/-}$ effectors alone groups was accompanied by significant changes to the gut architecture that was not observed in either of the groups in which T_{regs} were co-transferred. Significant increases in crypt length could be observed in the WT and SAP-$1^{-/-}$ effector alone groups (Figure 5.6B top row panels 1 and 2), whilst normal crypt lengths were observed in groups with T_{regs} co-injected (Figure 5.6B top row panels 3 and 4). Additionally large numbers of infiltrates could be observed in the effector alone groups whilst minimal infiltration was detected in the groups with T_{regs} co-injected. Finally loss of goblet cells was detected upon alcian blue staining in the effector alone groups (Figure 5.6B bottom row panels 1 and 2), which was not detected in the groups with T_{regs} co-injected (Figure 5.6B bottom rows panels 3 and 4).

Scores were generated from the histological data. Injection of WT and SAP-$1^{-/-}$ effectors alone gave scores indicative of severe colitis (3.0 ± 0.3 and 2.4 ± 0.8 respectively) (Figure 5.6C). In contrast colitis was prevented in the group with the wild type T_{regs} co-transferred with an average colitic score of 0.1 ± 0.1 $p=0.008$ (Figure 5.6C). The co-injection of SAP-$1^{-/-}$ T_{regs} at a 1 T_{reg}:2 Effector T cell ratio also significantly prevented the development of disease with an average colitic score of 1.0 ± 0.3 $p=0.008$ (Figure 5.6C). This experiment is representative of two experiments performed at this ratio. Thus SAP-$1^{-/-}$ T_{regs} are capable of preventing colitis. It remains possible that they function less efficiently as at a 1 T_{reg}:4 Effector T cell ratio, there was a more marked difference between the efficiency of WT and SAP-$1^{-/-}$ T_{reg} suppression (Figure 5.6D).

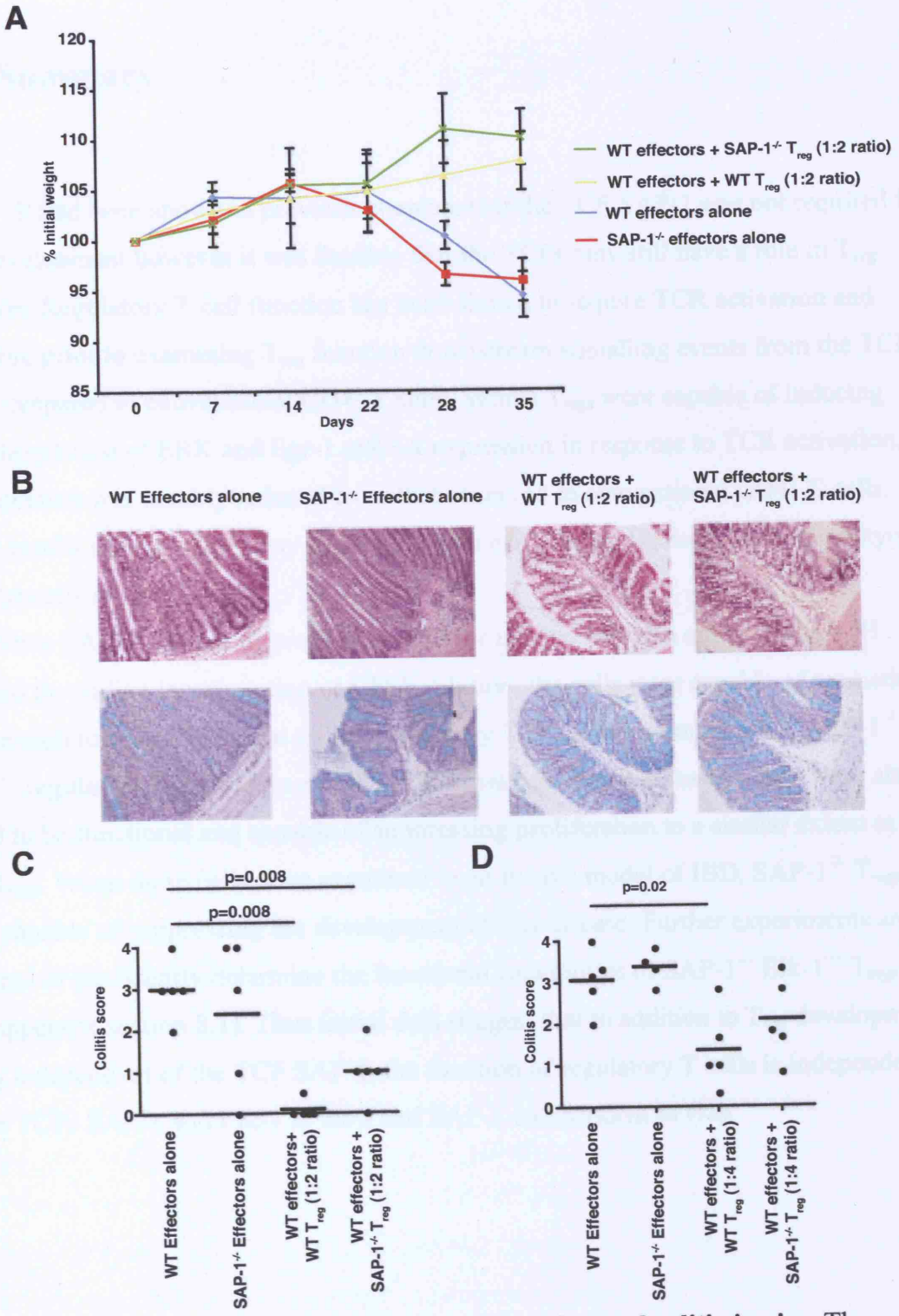


Figure 5.6. SAP-1$^{-/-}$ T_{reg} Suppression of experimental colitis *in vivo*. The colitis model of Inflamatory Bowel Disease (IBD) was used to assess function of T_{regs} *in vivo*. Animals were injected with naive effector T cells either alone or with T_{regs} from WT or SAP-1$^{-/-}$ animals. **A-C.** T_{regs} added at a 1 T_{reg}:2 Effector T cells ratio. **A.** Disease progression was monitored by measuring the weight of mice. Once mice lost 20% of their weight, as directed by our Ethics Committee guidelines, mice were sacrificed and the experiment terminated. **B.** Hisological analysis was performed to generate scores of colitis severity. Top row - H&E staining, bottom row- alcian blue staining. **C.** Colitis score at 1:2 ratio **D**. Colitis scores at 1:4 ratio

5.8 Summary

It had been shown in previous chapters that the TCF SAP-1 was not required for T_{reg} development however it was feasible that the TCFs may still have a role in T_{reg} function. Regulatory T cell function has been shown to require TCR activation and therefore prior to examining T_{reg} function downstream signalling events from the TCR were compared to conventional $CD4^+$ T cells. Whilst T_{regs} were capable of inducing phosphorylation of ERK and Egr-1 mRNA expression in response to TCR activation, the induction was notably reduced from that observed in conventional $CD4^+$ T cells. These results may go some way to providing an explanation for the anergic phenotype of these cells *in vitro*.

When SAP-$1^{-/-}$ T_{reg} cells were examined for *in vitro* function either through ^3H labelled thymidine incorporation or CFSE dilution, the cells were capable of mediating suppression to the same extent as WT regulatory T cells. Furthermore when SAP-$1^{-/-}$ Elk-$1^{-/-}$ regulatory T cells were examined in *in vitro* suppression assays they were also found to be functional and capable of suppressing proliferation to a similar extent as WT T_{regs}. When these cells were examined in an *in vivo* model of IBD, SAP-$1^{-/-}$ T_{regs} were capable of suppressing the development of this disease. Further experiments are required to confidently determine the functional capabilities of SAP-$1^{-/-}$ Elk-$1^{-/-}$ T_{regs} (see appendix section 8.1). Thus initial data suggest that in addition to T_{reg} development being independent of the TCF SAP-1, the function of regulatory T cells is independent of the TCFs SAP-1 and Elk-1 *in vitro* and SAP-1 independent *in vivo*.

6 Results – Defective ERK signalling and Regulatory T cell development

6.1 Abstract

The Ras-Raf-ERK cascade has shown to be important in positive selection, however few studies have addressed a role for this pathway in T_{reg} development. It has been proposed that T_{regs} may differentiate after positive selection has occurred. Therefore it is possible that ERK may be important for T_{reg} development as well as positive selection. Examination of mice expressing a dominant negative Raf transgene which display defective ERK signalling, demonstrated a role for Ras-Raf-ERK signalling in both positive selection and T_{reg} development. However DN Raf T_{regs} appear to be functional *in vitro* suggesting that once a cell has committed to the T_{reg} lineage, ERK signalling is not required for their subsequent function.

6.2 Introduction

T_{reg} development is independent of the TCF SAP-1 in contrast to the requirement for SAP-1 in positive selection. However T_{reg} development has been proposed not only to require interactions between the TCR and MHC but it has also been suggested that they develop subsequently to positive selection. I attempted to address whether there was an overlap between the signalling requirements of positive selection and regulatory T cell development and if so at what point the signals diverge. To do this I investigated the importance of ERK signalling in regulatory T cell development. As previously described, ERK signalling has been implicated in several immune processes including thymocyte positive selection (reviewed in Dong et al., 2002; Zhang and Dong, 2005). Here I used a dominant negative Raf (DN Raf) model to address the question of whether ERK signalling is required for T_{reg} development and function.

6.3 Thymocyte development in ERK1$^{-/-}$ mice

ERK1$^{-/-}$ mice had previously been reported to have a defect in positive selection with approximately a 50% reduction in both CD4^{+} and CD8^{+} SP thymocytes (Pages et al., 1999), similar to that described in the SAP-1$^{-/-}$ animals. Regulatory T cell development was not examined in these animals. Therefore it was possible that these animals would provide insight into not only whether ERK was required for T_{reg} development but might also identify the point at which the signals for positive selection and T_{reg} development diverge. ERK1$^{-/-}$ mice were obtained from J. Pouyssegur.

Total thymocyte cell extracts were prepared from equivalent cell numbers of WT and ERK1$^{-/-}$ thymocytes and analysed by immunoblot for ERK expression. As expected both ERK1 and ERK2 could clearly be detected in WT thymocytes (Figure 6.1A lanes 1 to 3), whilst only ERK2 could be detected in thymocyte isolated from ERK1$^{-/-}$ animals (Figure 6.1A lanes 4 to 6).

Thymocyte development was then assessed in these animals by the expression of CD4 and CD8. Surprisingly only a minimal reduction in SP thymocytes was observed, with the proportion of CD4^{+} SP being reduced from 9.6% ± 0.3 in WT mice to 7.8% ± 0.2 in ERK1$^{-/-}$ mice (n=3, p=0.01) (Figure 6.1B). Analysis of TCRβ showed that there was no difference in the levels of TCR^{hi} expression in WT and ERK1$^{-/-}$ mice (Figure 1C). Gating on TCR^{hi} failed to reveal a significant decrease in the proportion of CD4^{+} and CD8^{+} SP thymocytes in ERK1$^{-/-}$ mice (Figure 6.1D). These data are in contrast to the positive selection defect previously described (Pages et al., 1999). This observation has since been confirmed in a paper examining the effects of a conditional ERK2 deletion (Fischer et al., 2005). Despite the lack of a severe defect in positive selection, regulatory T cell development was examined by the expression of CD25 on CD4^{+} gated cells. No difference could be detected between the proportion of CD4^{+}CD25^{+} T cells in WT and ERK1$^{-/-}$ mice (Figure 6.1E).

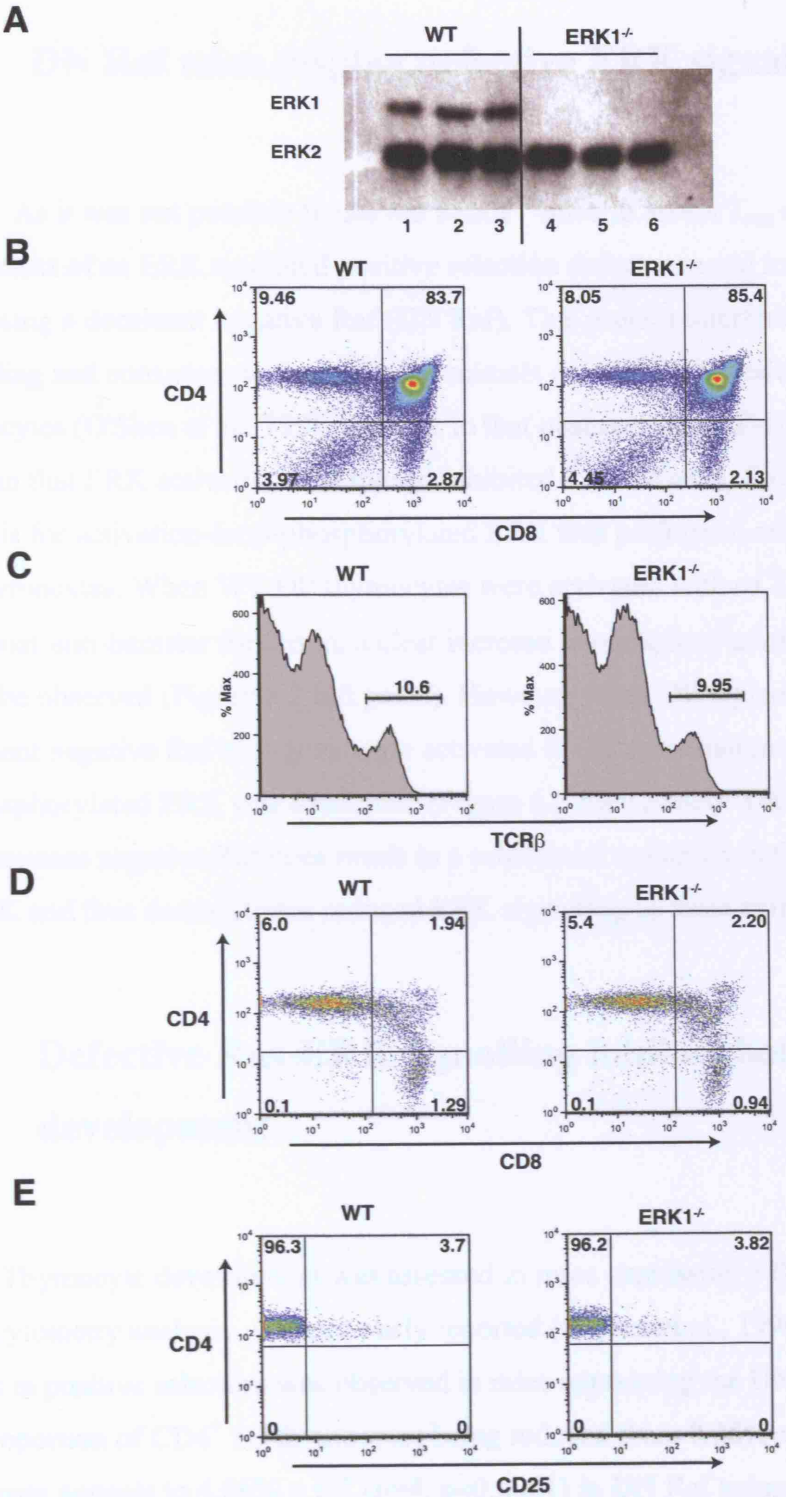


Figure 6.1. ERK1$^{-/-}$ mice display no defect in thymocyte development. A. Immunoblot analysis to show ERK1 protein depleted in ERK1$^{-/-}$ mice. **B-E.** Thymocyte development in ERK1$^{-/-}$ mice was assessed by flow cytometry. **B.** Analysis of CD4 and CD8 expression. **C.** Expression of TCRβ on total thymocytes. **D.** CD4 and CD8 expression on TCRβ^{hi} gated thymocytes. Proportion of total thymocytes displayed inset. **E.** CD25 expression on CD4^{+} gated thymocytes.

6.4 DN Raf mice display defective ERK signalling

As it was not possible to use the ERK1$^{-/-}$ mice to assess T_{reg} development within the context of an ERK mediated positive selection defect we used transgenic mice expressing a dominant negative Raf (DN Raf). This protein interferes with Ras-ERK signalling and consistent with this, these animals exhibit a 50% reduction in SP thymocytes (O'Shea et al., 1996); similar to that observed in SAP-1$^{-/-}$ animals. To confirm that ERK activation was indeed inhibited in these cells, flow cytometry analysis for activation-loop-phosphorylated ERK was performed on TCR crosslinked DP thymocytes. When WT DP thymocytes were activated with αCD3 and crosslinked with goat-anti-hamster for 2 min, a clear increase in phosphorylation levels of ERK could be observed (Figure 6.2 left panel). However when DP thymocytes expressing the dominant negative Raf transgene were activated in the same manner, very little increase in phosphorylated ERK was detectable (Figure 6.2 right panel). Thus the expression of the dominant negative Raf does result in a substantial reduction in the phosphorylation of ERK and thus demonstrates reduced ERK signalling in these animals.

6.5 Defective Ras-ERK signalling inhibits both SP and T_{reg} development

Thymocyte development was assessed in mice expressing a DN Raf transgene by flow cytometry analysis. As previously reported (O'Shea et al., 1996) a significant defect in positive selection was observed in mice expressing the DN Raf transgene with the proportion of CD4$^+$ SP thymocytes being reduced from 8.26% ± 0.3 in WT littermate animals to 4.06% ± 0.2 (n=4, p<0.0001) in DN Raf animals (Figure 6.3A). Likewise the proportions of CD8$^+$ SP thymocytes were reduced from 1.7% ± 0.1 in WT littermates to 0.8% ± 0.1 (n=4, p=0.001) in DN Raf animals. This defect was similar to that observed in the SAP-1$^{-/-}$ animals. In contrast to the SAP-1$^{-/-}$ animals, the proportion of CD4$^+$CD25$^+$ T cells was only marginally enhanced in DN Raf mice from 4.0% ± 0.2 in WT littermates to 5.1% ± 0.5 (n=4, p=0.05) in DN Raf mice (Figure 6.3B).

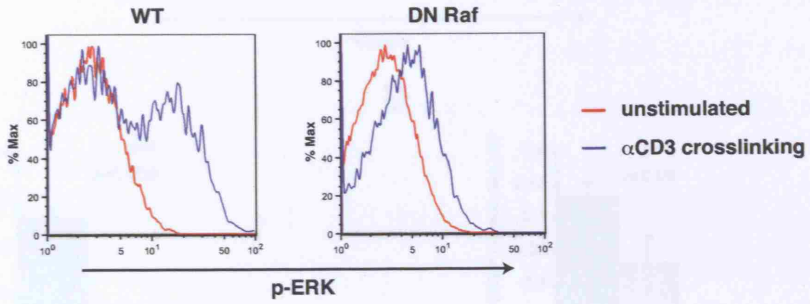


Figure 6.2. Mice expressing a DN Raf transgene display defective ERK activation. Flow cytometry analysis of activation-loop-phosphorylation of ERK in WT and DN Raf mice. DP thymocytes were activated with αCD3 and then crosslinked for 2 min.

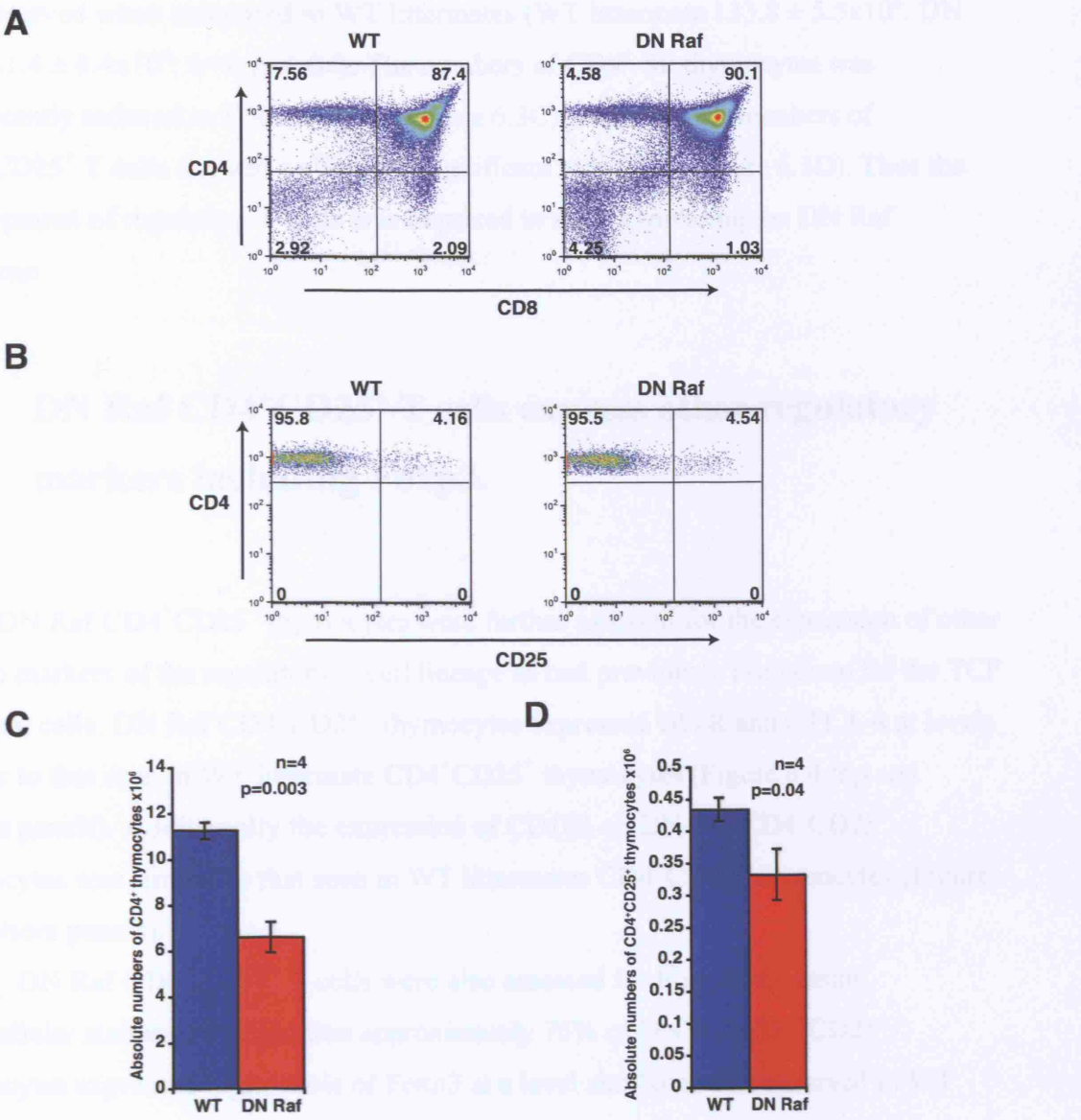


Figure 6.3. DN Raf mice display defects in thymocyte development. A. and **B.** Thymocyte development in WT and DN Raf mice assessd by flow cytometry. **A.** Analysis of CD4 and CD8 expression. **B.** CD25 expression on $CD4^+$ gated thymocytes. **C.** Absolute numbers of $CD4^+$ thymocytes. **D.** Absolute numbers of $CD4^+CD25^+$ thymocytes.

In mice expressing the DN Raf transgene a slight increase in thymic cellularity was observed when compared to WT littermates (WT littermate 133.8 ± 5.5x10^6; DN Raf 161.4 ± 8.4x10^6; n=4, p=0.04). The numbers of $CD4^+$ SP thymocytes was significantly reduced in DN Raf mice (Figure 6.3C). The absolute numbers of $CD4^+CD25^+$ T cells showed a slight but significant reduction (Figure 6.3D). Thus the development of regulatory T cells was impaired in mice expressing the DN Raf transgene

6.6 DN Raf $CD4^+CD25^+$ T cells express other regulatory markers including Foxp3.

DN Raf $CD4^+CD25^+$ thymocytes were further assessed for the expression of other known markers of the regulatory T cell lineage as had previously been done for the TCF deficient cells. DN Raf $CD4^+CD25^+$ thymocytes expressed GITR and CTLA-4 at levels similar to that seen in WT littermate $CD4^+CD25^+$ thymocytes (Figure 6.4 top and middle panels). Additionally the expression of CD103 on DN Raf $CD4^+CD25^+$ thymocytes was similar to that seen in WT littermates $CD4^+CD25^+$ thymocytes (Figure 6.4 bottom panels).

DN Raf $CD4^+CD25^+$ T cells were also assessed for Foxp3 expression. Intracellular staining revealed that approximately 75% of DN Raf $CD4^+CD25^+$ thymocytes expressed high levels of Foxp3 at a level similar to that observed in WT littermate cells. (Figure 6.5 top panels). Similar results were observed when DN Raf $CD4^+CD25^+$ T cells from spleen and lymph nodes were examined (Figure 6.5 middle and bottom panels).

Examination of thymocyte profiles incorporating intracellular Foxp3 staining reflected data collected for the development of $CD4^+CD25^+$ T cells. DN Raf mice only displayed a marginal enhancement of $CD4^+Foxp3^+$ thymocytes unlike that observed for SAP-$1^{-/-}$ mice (4.5% ± 0.3 in WT littermate animals; 5.6% ± 0.5 DN Raf animals; n=6) (Figure 6.6A). However when the absolute numbers of $CD4^+Foxp3^+$ thymocytes were calculated, as was seen for the numbers of $CD4^+CD25^+$ thymocytes in DN Raf mice, the absolute numbers of $CD4^+Foxp3^+$ thymocytes showed a slight but significant reduction (Figure 6.6B). These data demonstrate that the Ras-Raf-ERK signalling pathway is required for T_{reg} development in addition to positive selection.

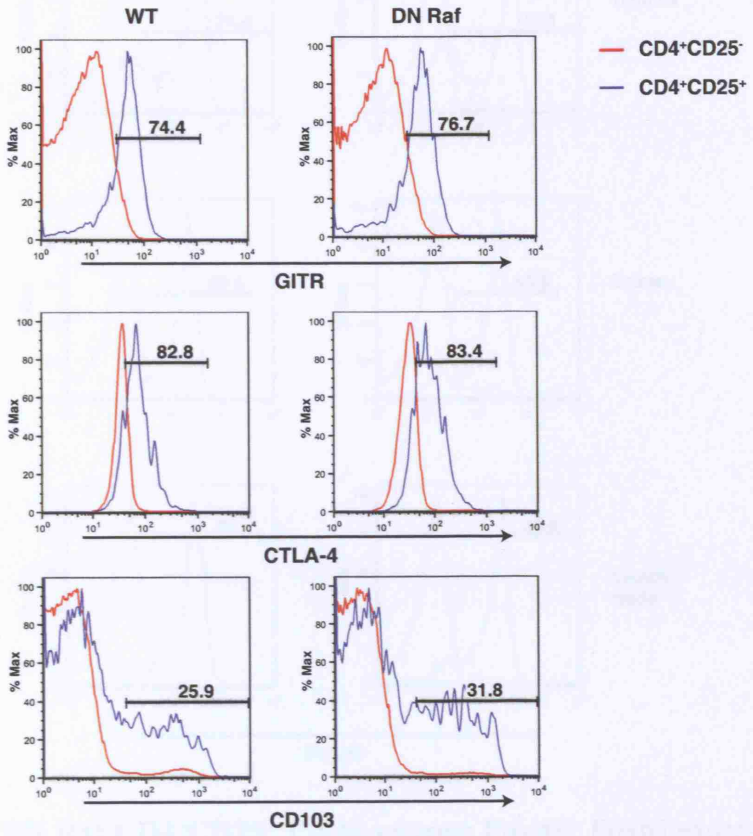


Figure 6.4. DN Raf $CD4^+CD25^+$ thymocytes express other regulatory markers. Expression of regulatory markers was assessed by flow cytometry analysis. GITR - top panels, CTLA-4 - middle panels and CD103 - bottom panels. No difference was observed between WT and DN Raf thymocytes.

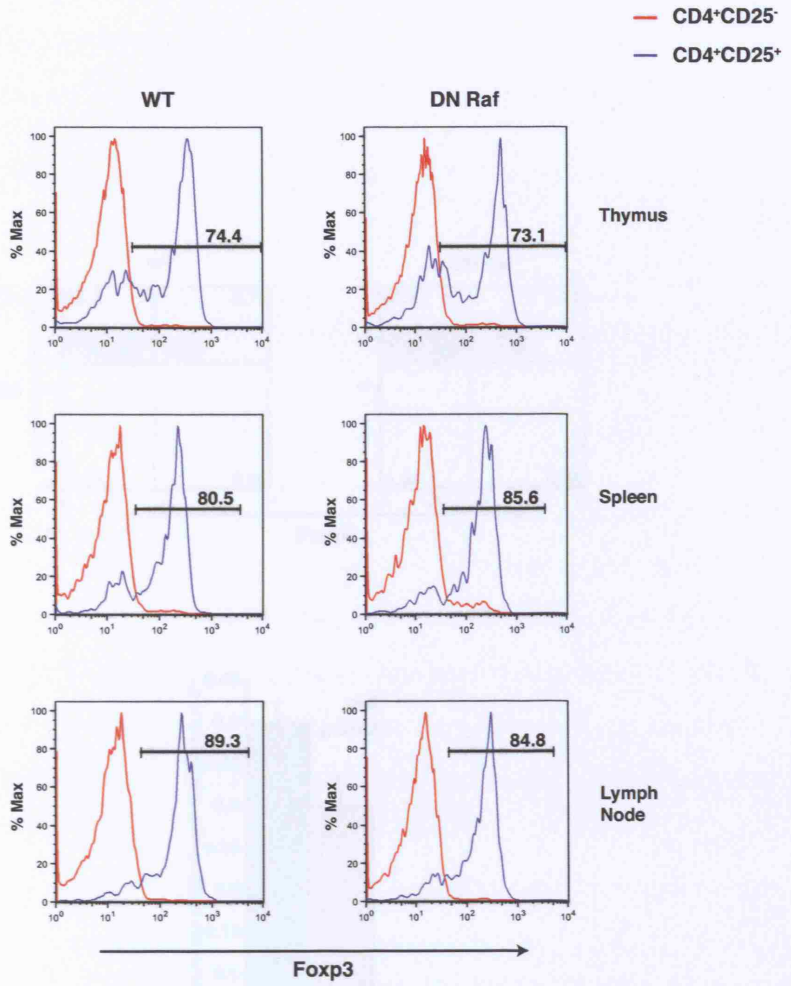


Figure 6.5. DN Raf $CD4^+CD25^+$ T cells express Foxp3. Foxp3 expression was assessed on a per cell basis by intracellular staining. The majority of both WT and DN Raf $CD4^+CD25^+$ T cells express Foxp3. Cells isolated from: thymus (top panels); spleen (middle panel); and lymph node (bottom panels).

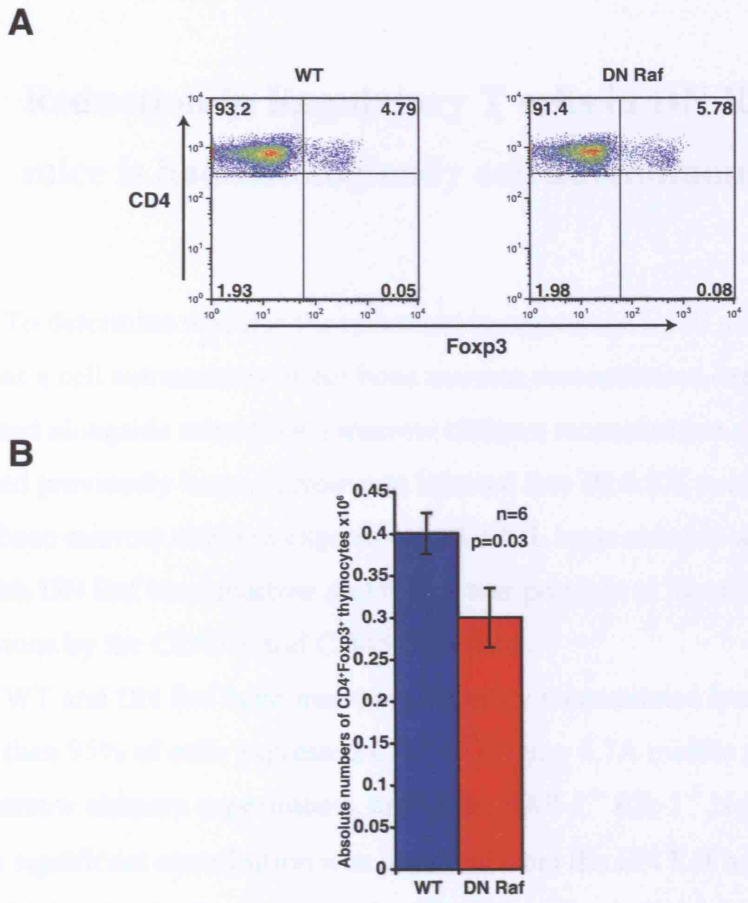


Figure 6.6. DN Raf mice display defects in regulatory T cell development. **A.** Flow cytometry analysis of Foxp3 expression on $CD4^+$ gated thymocytes in mice expressing a DN Raf transgene. **B.** Absolute numbers of $CD4^+Foxp3^+$ thymocytes.

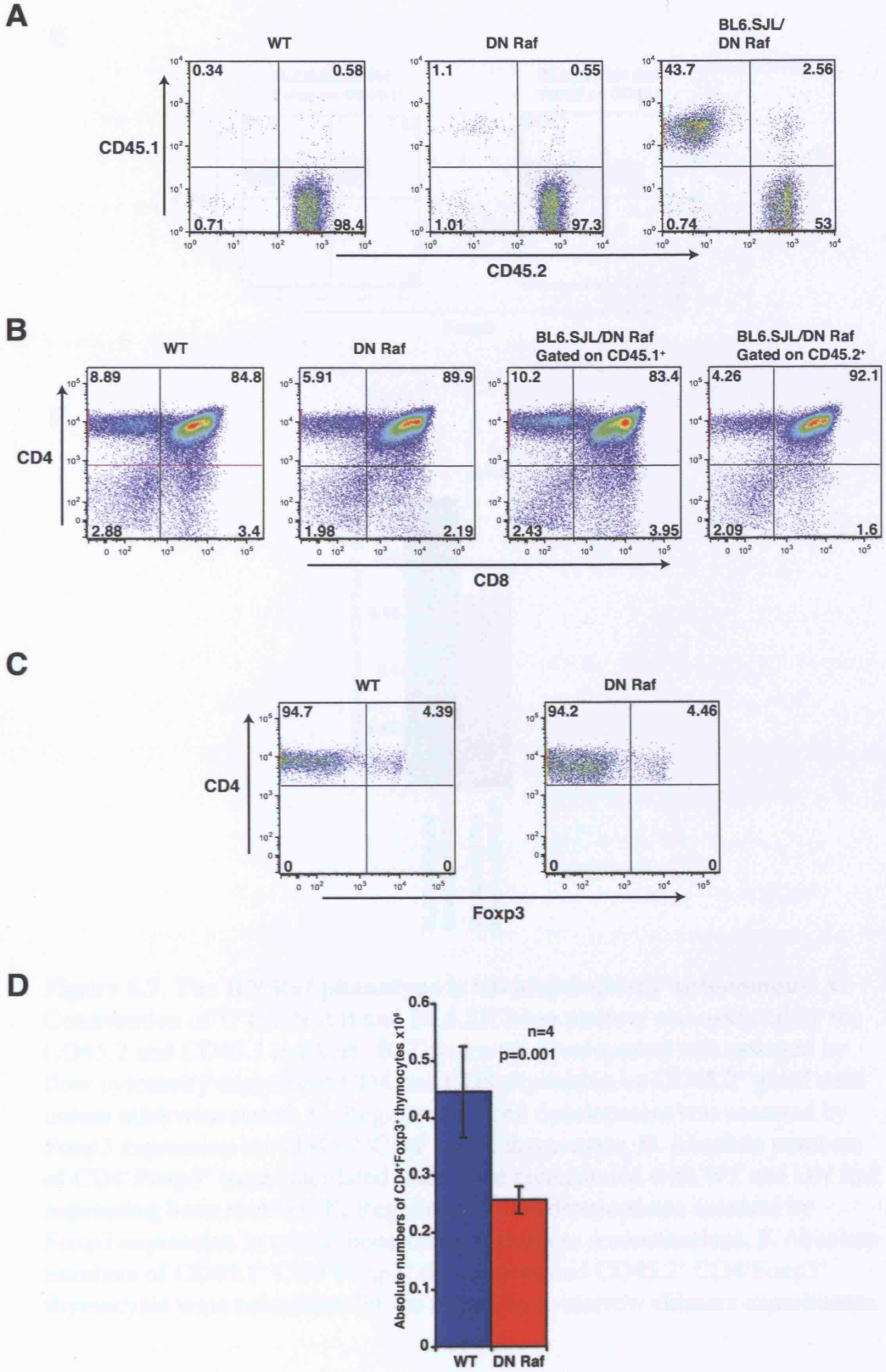
This contrasts with the data from SAP-1 deficient animals where only positive selection required SAP-1 signalling. Taken together these data suggest that the signals for T_{reg} development may act via a target of ERK signalling but this target is not SAP-1. Thus the point at which the signals for positive selection and T_{reg} development diverge may be at the level of ERK activation, this possibility will be expanded on in the discussion (see section 7.5.3).

6.7 Reduction in Regulatory T cells in DN Raf expressing mice is haematologically cell autonomous

To determine whether the reduction in regulatory T cell numbers in the DN Raf mice was a cell autonomous affect bone marrow reconstitution experiments were performed alongside mixed bone marrow chimera reconstitution experiments. As described previously bone marrow was injected into BL6.SJL recipients, and for the mixed bone marrow chimera experiment BL6.SJL bone marrow was mixed at a 1:1 ratio with DN Raf bone marrow and thus it was possible to identify the relevant populations by the CD45.1 and CD45.2 markers.

WT and DN Raf bone marrow efficiently reconstituted lymphopenic animals as greater than 95% of cells expressed CD45.2 (Figure 6.7A middle panel). In the mixed bone marrow chimera experiments, unlike the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ experiments (see 4.10), a significant contribution was observed from the DN Raf bone marrow in that 67% ± 6.3 (n=4) of cells expressed the CD45.2 marker indicating that they derived from the DN Raf bone marrow (Figure 6.7A panel right panel).

Reconstitution with DN Raf bone marrow resulted in approximately 50% reduction in the proportion of SP thymocytes when compared to WT animals. The proportion of $CD4^+$ SP T cells was reduced from 9.2% ± 1.0 in WT reconstituted animals to 5.4% ± 0.3 in animals reconstituted with DN Raf bone marrow (p=0.01, n=4) (Figure 6.7B). When mice were reconstituted with BL6.SJL/DN Raf mixed bone marrow, gating on $CD45.1^+$ revealed a WT profile with 10.4% ± 1.6 $CD4^+$ SP thymocytes (Figure 6.7B). However the DN Raf thymocytes (gated on $CD45.2^+$) displayed a substantial reduction in SP thymocytes with $CD4^+$ SP thymocytes being reduced to 4.6% ± 0.1 (Figure 6.7B). Thus not only is the positive selection defect


A
WT
DN Raf
BL6.SJL/
DN Raf
CD45.1
0.34
0.58
0.71
98.4
1.1
0.55
1.01
97.3
43.7
2.56
0.74
53
CD45.2
B
WT
DN Raf
BL6.SJL/DN Raf
Gated on CD45.1+
BL6.SJL/DN Raf
Gated on CD45.2+
CD4
8.89
84.8
2.88
3.4
5.91
89.9
1.98
2.19
10.2
83.4
2.43
3.95
4.26
92.1
2.09
1.6
CD8
C
WT
DN Raf
CD4
94.7
4.39
0
0
94.2
4.46
0
0
Foxp3
D
n=4
p=0.001
Absolute numbers of CD4+Foxp3+ thymocytes x10⁶
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
DN Raf

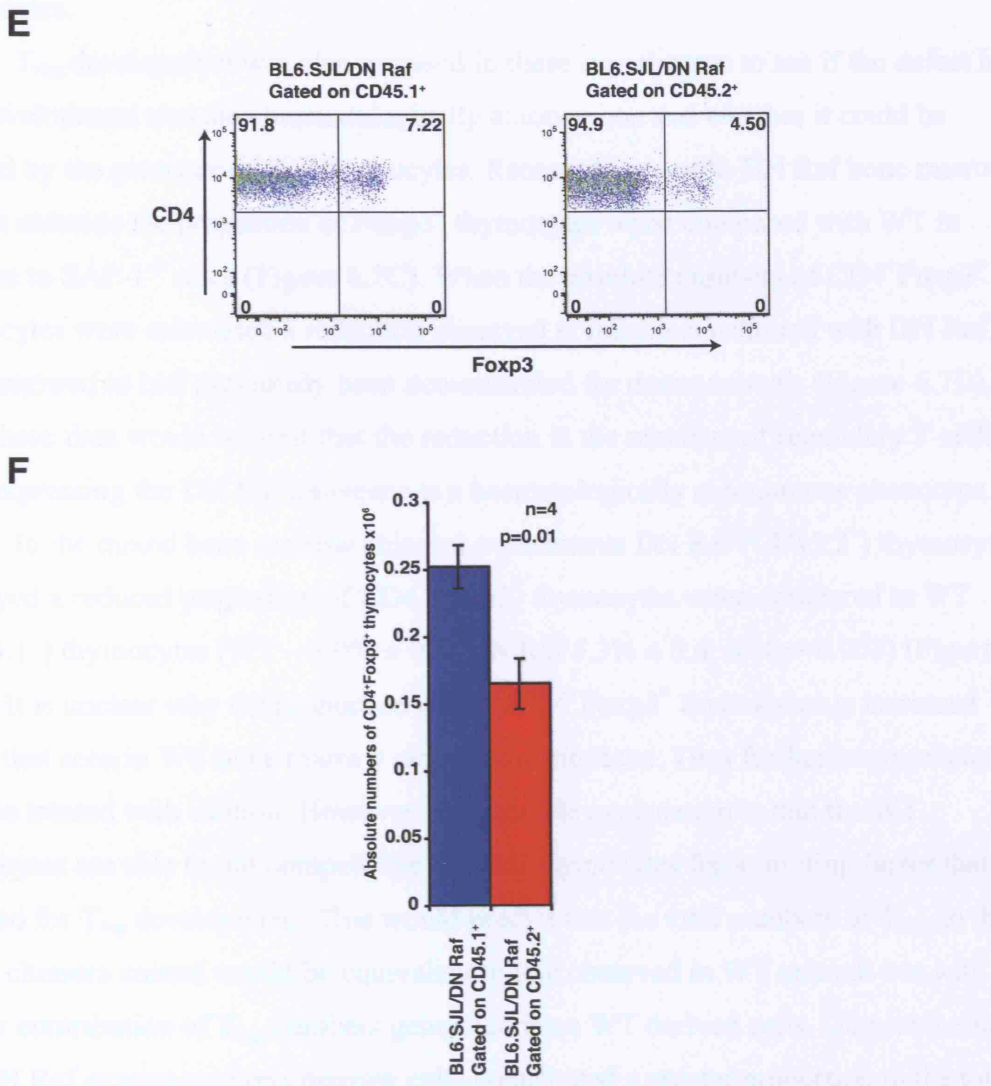


Figure 6.7. The DN Raf phenotype is haematologically autonomous. A. Contribution of WT, DN Raf and BL6.SJLbone marrow was assessed by the CD45.2 and CD45.1 markers. **B.** Thymocyte development was assessed by flow cytometry analysis of CD4 and CD8 expression on CD45.2$^+$ gated cells unless otherwise stated. **C.** Regulatory T cell development was assessed by Foxp3 expression on CD45.2$^+$CD4$^+$ gated thymocytes. **D.** Absolute numbers of CD4$^+$Foxp3$^+$ were calculated from mice reconsituted with WT and DN Raf expressing bone marrow. **E.** Regulatory T cell development assessed by Foxp3 expression in mixed bone marrow chimera reconstitutions. **F.** Absolute numbers of CD45.1$^+$ CD4$^+$Foxp3$^+$ thymocytes and CD45.2$^+$ CD4$^+$Foxp3$^+$ thymocytes were calculated for the mixed bone marrow chimera experiments.

haematologically autonomous but also it cannot be rescued by the presence of WT thymocytes.

T_{reg} development was also assessed in these experiments to see if the defect in T_{reg} development was also haematologically autonomous and whether it could be rescued by the presence of WT thymocytes. Reconstitution with DN Raf bone marrow did not enhance the proportion of $Foxp3^+$ thymocytes when compared with WT in contrast to SAP-$1^{-/-}$ mice (Figure 6.7C). When the absolute numbers of $CD4^+Foxp3^+$ thymocytes were calculated a reduction observed in mice reconstituted with DN Raf bone marrow, as had previously been demonstrated for donor animals (Figure 6.7D). Thus these data would suggest that the reduction in the numbers of regulatory T cells in mice expressing the DN Raf transgene is a haematologically autonomous phenotype.

In the mixed bone marrow chimera experiments DN Raf ($CD45.2^+$) thymocytes displayed a reduced proportion of $CD4^+Foxp3^+$ thymocytes when compared to WT ($CD45.1^+$) thymocytes (WT – 8.0% ± 0.4; DN Raf 5.3% ± 0.4; n=4,p=0.003) (Figure 6.7E). It is unclear why the proportion of WT $CD4^+Foxp3^+$ thymocytes is increased above that seen in WT bone marrow alone reconstitutions. Thus further interpretation must be treated with caution. However one possible explanation is that the WT thymocytes are able to out compete the DN Raf thymocytes for a limiting factor that is required for T_{reg} development. This would predict that the total numbers of T_{regs} in the mixed chimera animal would be equivalent to that observed in WT animals but with a greater contribution of T_{reg} numbers generated from WT derived cells. Despite the fact that DN Raf expressing bone marrow cells contributed a greater proportion to the total thymocyte pool, DN Raf ($CD45.2^+$) thymocytes in the mixed bone marrow chimera experiments displayed a significant reduction in absolute numbers of $CD4^+Foxp3^+$ thymocytes when compared with WT bone marrow ($CD45.1^+$) derived cells (Figure 6.7F). However the absolute numbers of T_{regs} in the chimera animals was approximately $0.42 \times 10^6 \pm 0.03$, which is similar to that previously observed for WT animals.

These data suggest that the reduction in $CD4^+Foxp3^+$ thymocytes observed in mice expressing a dominant negative Raf transgene cannot be rescued by the presence of WT thymocytes. Thus these data demonstrate that the Ras-ERK signalling pathway is required for both positive selection and T_{reg} development.

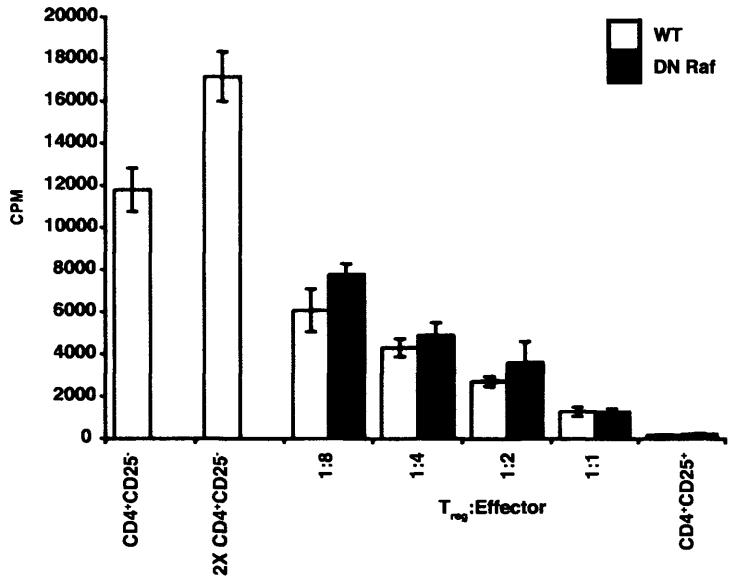


Figure 6.8. DN Raf $CD4^+CD25^+$ T cells are functional *in vitro*. $CD4^+CD25^-$ T cells isolated from WT lymph nodes were incubated with increasing amounts of $CD4^+CD25^+$ T cells isolated fromWT or DN Raf lymph nodes. Cells were incubated for 72 hours and proliferation was assessed by ^3H labelled thymidine incorporation.

6.8 Function of DN Raf T_{regs}

To test whether Raf-ERK signalling is required for suppression by T_{regs} *in vitro* suppression assays were performed using $CD4^+CD25^+$ T cells isolated from WT and DN Raf animals. WT effector cells were sorted as $CD4^+CD25^-$ T cells from lymph nodes and activated in the presence of αCD3 and APCs. These cells were co-cultured with the $CD4^+CD25^+$ T cells at varying ratios typically ranging from 1 T_{reg}:1 Effector cell down to a 1 T_{reg}:8 Effector cells. Proliferation was determined by the incorporation of ^3H labelled thymidine. When cells were cultured alone under activating conditions $CD4^+CD25^-$ T cells proliferated whilst both WT and DN Raf $CD4^+CD25^+$ T cells did not (Figure 6.8). Addition of WT $CD4^+CD25^+$ T cells in increasing numbers to the $CD4^+CD25^-$ T cell cultures resulted in suppression of proliferation in a dose dependent fashion (Figure 6.8). Addition of DN Raf $CD4^+CD25^+$ T cells also resulted in a dose dependent reduction in proliferation (Figure 6.8). At the point were a 1:1 ratio was established proliferation was suppressed by approximately 80% whether WT or DN Raf $CD4^+CD25^+$ T cells had been added. This was unlikely to be as a result of competition for limiting factors in the cell culture medium as the further addition of an equal number of $CD4^+CD25^-$ effectors to $CD4^+CD25^-$ T cell cultures resulted in increased proliferation (Figure 6.8). The suppression of WT effectors was equivalent at all ratios between WT and DN Raf T_{regs}. Thus whilst ERK signalling is required for the development of regulatory T cells it is not required for suppressive function *in vitro*.

6.9 Implication of ERK signalling in T_{reg} development and function

The DN Raf data have implicated the Ras-ERK signalling pathway in T_{reg} development but not function. However it was not possible to use the ERK-$1^{-/-}$ to directly implicate ERK signalling in T_{reg} development or function. Therefore I examined other *in vitro* assays that may allow an insight into the function of ERK in T_{reg} development and function.

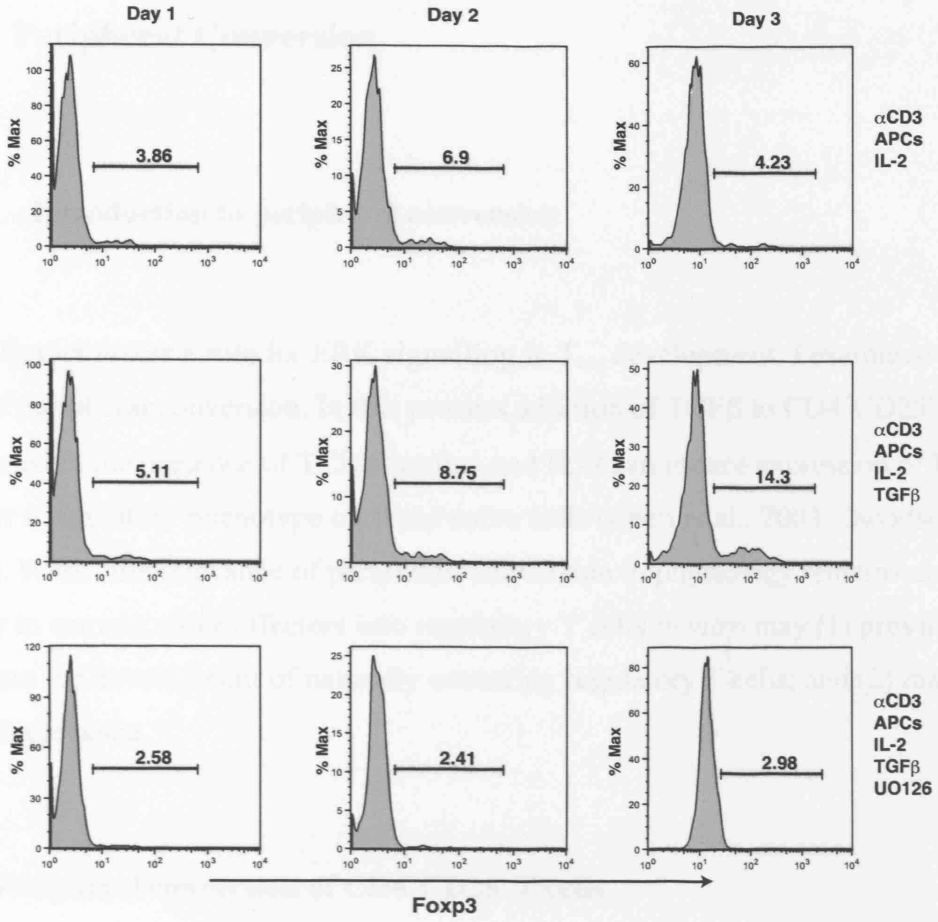


Figure 6.9. Peripheral conversion is ERK dependent. Cells were cultured over 3 days in either: αCD3, APCs and IL-2 (top row); αCD3, APCs, IL-2 and TGFβ (middle row); or αCD3, APCs, IL-2, TGFβ and UO126 (bottom row). Foxp3 expression was assessed by intracellular staining.

6.9.1 Peripheral Conversion

6.9.1.1 Introduction to peripheral conversion

To further assess a role for ERK signalling in T_{reg} development, I examined the process of peripheral conversion. In this process addition of TGFβ to $CD4^+CD25^-$ T cells cultured in the presence of TCR stimulus and IL-2 can induce expression of Foxp3 and confer a regulatory phenotype on these naïve cells (Chen et al., 2003; Davidson et al., 2007). Whilst the relevance of peripheral conversion to physiology remains unclear, the ability to convert naïve effectors into regulatory T cells *in vitro* may (1) provide insights into the development of naturally occurring regulatory T cells; and (2) may be of clinical relevance.

6.9.1.2 Peripheral conversion of $CD4^+CD25^-$ T cells

To establish the peripheral conversion system, $CD4^+CD25^-$ T cells were isolated from WT lymph nodes and cultured under conversion inducing conditions. Foxp3 expression, as determined by intracellular staining, was used to assess the conversion to regulatory type T cells. Culturing naïve effector T cells with αCD3, APCs and exogenous IL-2 did not induce Foxp3 expression at any time point (Figure 6.9 top row). However activation in the presence of TGFβ increased the number of cells expressing Foxp3 (Figure 6.9 middle row). This is in accordance with previously published data (Davidson et al., 2007). The addition of the MEK inhibitor UO126 resulted in an inhibition of Foxp3 induction (Figure 6.9 bottom row). These results suggest that peripheral conversion is ERK-dependent. It is unlikely that UO126 acts to block the expansion of the small numbers of Foxp3 expressing cells present at the start of the experiment since previous work has shown TGFβ does not induce the expansion of naturally occurring T_{regs} (Chen et al., 2003) however, the effects on proliferation were not directly assessed in these experiments. These results indicate that ERK signalling is required during the process of peripheral conversion. Attempts to use DN Raf and SAP-

$1^{-/-}$ naïve cells have as yet not produced conclusive results (see Appendix section 8.2), and thus it is not possible to say if this process is akin to thymic development of T_{regs}. Therefore at present it is not possible to use these results to infer anything with regard to thymic development of T_{regs} and thus the process of peripheral conversion needs to be viewed as a distinct process that requires ERK signalling.

6.9.2 Pre activation of T_{regs} requires ERK signalling

Being unable to use an *in vivo* model of suppression to further address the role of ERK signalling in T_{reg} function, the requirements for pre-activation of regulatory T cells was examined. Recent studies have shown that pre-activation of $CD4^+CD25^+$ T cells prior to co-culturing them with $CD4^+CD25^-$ T cells increased their suppressive capabilities (Thornton et al., 2004). Since ERK signalling and IL-2 signalling have been linked (Genot et al., 1996) I investigated whether ERK signalling was important during this pre-activation stage and thus indicate a role for ERK signalling in T_{reg} function.

$CD4^+CD25^+$ T cells were isolated from lymph node and pre-cultured with αCD3, αCD28 and IL-2 for 3 days. The cells were then washed before adding to an *in vitro* suppression assay alongside freshly isolated $CD4^+CD25^+$ T cells. As previously described, when freshly isolated $CD4^+CD25^+$ T cells were added to cultures of $CD4^+CD25^-$ T cells suppression of proliferation could be observed in a dose dependent fashion (Figure 6.10A). However when "pre-activated" $CD4^+CD25^+$ T cells were added after three days of pre-culture with αCD3, αCD28 and IL-2, the suppressive activity of these cells was enhanced, agreeing with published observations (Thornton et al 2004). Suppression was observed even at a ratio of 1 T_{reg}: 32 Effector cells in contrast to freshly isolated T_{regs} that were unable to suppress at this ratio (Figure 6.10B). If the $CD4^+CD25^+$ T cells were additionally cultured in the presence of UO126, then this increase in suppressive ability at low ratios of $CD4^+CD25^+$ T cells was lost and the "pre-activated" T_{regs} behaved similarly to freshly isolated T_{regs} (Figure 6.10B). Whilst the proliferation was not as robust as in previous suppression assays, these preliminary data (representative of two separate experiments) suggests that inhibition of MEK1 activity can affect the ability to pre-activate regulatory T cells.

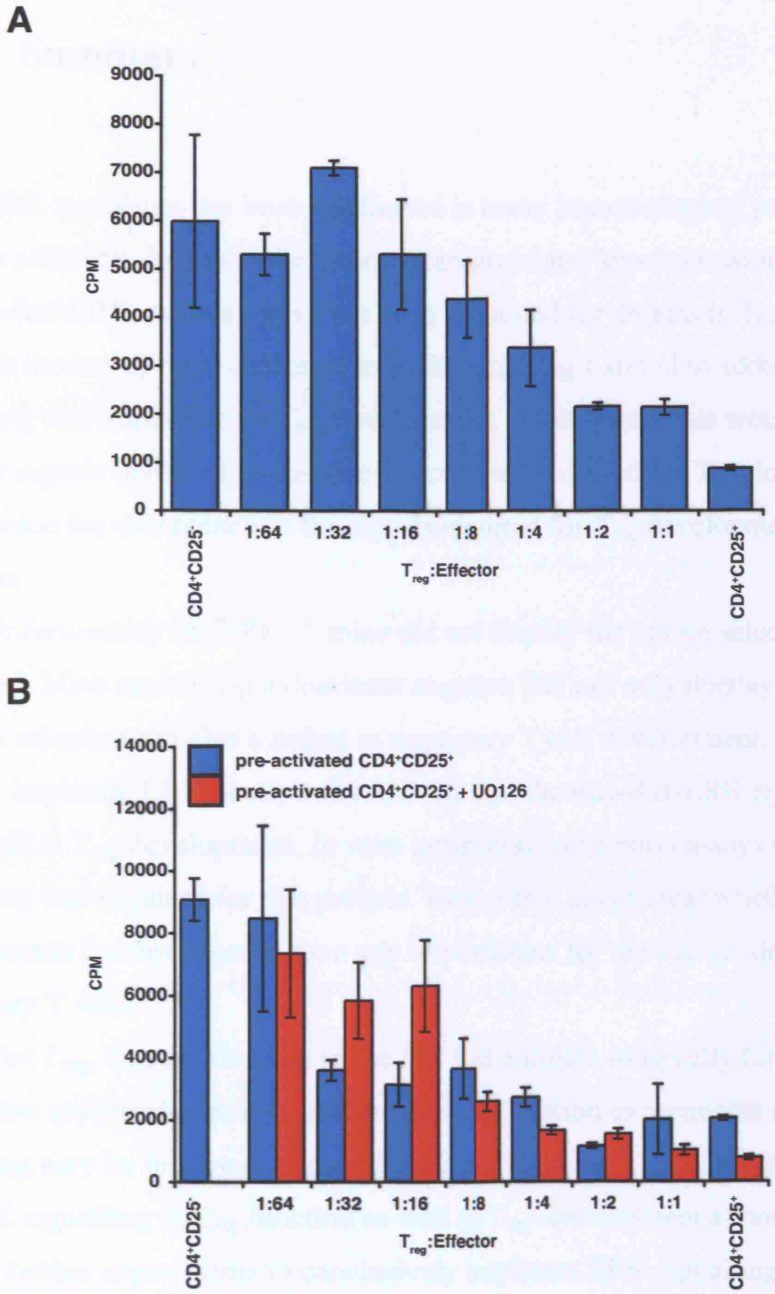


Figure 6.10. Pre-activation of CD4$^+$CD25$^+$ T cells requires ERK signalling. *In vitro* suppression assays, with proliferation measured by ^{3}H thymidine incoportation. **A.** *In vitro* suppression assay with freshly isolated WT CD4$^+$CD25$^+$ T cells. **B.** CD4$^+$CD25$^+$ T cells pre-cultured with αCD3, αCD28 and IL-2, and where stated UO126, for 72 hours and then added to an *in vitro* suppression assay. Results are average of triplicate samples, representative of two experiments.

6.10 Summary

ERK signalling has been implicated in many immunological processes, including positive selection, however the various transgenic and knockout animals with defects in the Ras-Raf-ERK pathway have not been examined for defects in T_{reg} development. Through the use of mice defective in ERK signalling I aimed to address whether ERK signalling was important for T_{reg} development. Additionally this would also address whether signals involved in positive selection are required for T_{reg} development and if so, examine the divergence of the signals required for T_{reg} development and positive selection.

Unfortunately the $ERK1^{-/-}$ mice did not display the severe selection phenotype as predicted. Mice expressing a dominant negative Raf not only displayed a defect in positive selection but also a defect in regulatory T cell development. Whilst this did not directly implicate ERK itself, it did indicate that the Ras-Raf-ERK signalling pathway is important in T_{reg} development. *In vitro* peripheral conversion assays showed that ERK signalling was required for this process. However it is not clear whether the requirements for this process have any implications for the thymic development of regulatory T cells.

The T_{regs} that did develop in the DN Raf animals were fully functional as assessed by *in vitro* suppression assays. Although pre-activation experiments suggest that ERK signalling may be important during the pre-activation of T_{regs}. Thus there may be a role for ERK signalling in T_{reg} function as well as T_{reg} development although this will require further experiments to conclusively implicate ERK signalling in T_{reg} function.

7 Discussion

7.1 Summary

The ternary complex factors (TCFs) are Ets domain transcription factors that have been shown to be direct targets of MAPK signalling cascades. Previous data from transgenic mouse studies have shown that MAPK signalling is involved in many cellular processes including proliferation, differentiation and apoptosis. Within the immune system MAPK signalling has been shown to be critical for early thymocyte development and during positive and negative selection.

The TCF SAP-1 has been shown to be important during thymocyte positive selection. Furthermore, TCF target genes including Egr-1 have been identified as critical mediators of this process. In this thesis I present data generated from the use of animal models deficient in components of the SRF network including SAP-1, Elk-1, Net and SRF itself. Experiments were designed to advance our understanding of the role of these transcription factors in regulatory T cell development and function. Additionally, mice expressing a dominant negative Raf (DN Raf) were used to further demonstrate that the Ras-ERK signalling pathway does play a role in regulatory T cell development but that this does not involve signalling to the TCF SAP-1.

7.2 Expression of TCF proteins

Previous studies have shown that loss of SAP-1 results in a severe defect in the positive selection process of thymocyte development (Costello et al., 2004), whilst Elk-$1^{-/-}$ and $Net^{\delta/\delta}$ mice exhibited no obvious immune phenotype (Cesari et al., 2004; Ayadi et al., 2001). However all three members of the family are widely expressed within immune cells (J.W. unpublished observations) which may represent some level of functional redundancy within certain immune cells. Within T cell populations SAP-1 is the predominant TCF, followed by Net and then Elk-1. The relative expression levels of

the different TCFs are similar throughout the thymocyte sub-populations. TCF mRNAs are expressed at lower levels within the DN population. It is possible that this reflects a requirement for TCF signalling only at later stages of development. Further work will be necessary to examine TCF expression within different DN sub-populations.

7.3 TCF compensation

Removal of Elk-1 on the SAP-$1^{-/-}$ background exacerbated the positive selection defect observed in the SAP-$1^{-/-}$ mice, suggesting that there may be some degree of redundancy between the two proteins during positive selection. It was also possible that upon deletion of one TCF the expression of the remaining TCFs was increased as a compensatory mechanism. Examination of TCF mRNA in DP thymocytes did not reveal any changes in TCF expression upon deletion of one or more TCFs, although it is possible that the activity of these proteins is altered. Thus it would appear that for some processes SAP-1 and Elk-1 are functionally redundant. Depletion of Net however did not appear to be functionally redundant in positive selection. Whilst Net is homologous to SAP-1 and Elk-1, it has been shown to have a reduced ability to bind to TCF target promoters under certain conditions (Giovane et al., 1994; Price et al., 1995). This may explain the lack of functional redundancy between Net and the other TCFs observed in positive selection.

7.4 TCFs in early development

Analysis of DN populations in TCF-deficient animals suggests that TCFs do not play a role during DN development (Costello et al., 2004 and unpublished observations).

In contrast, the Ras-ERK pathway clearly is involved in β-selection. $ERK2^{f/f}$ pLCK-Cre^{+} mice displayed reduced numbers of DP thymocytes consistent with a partial block in the DN-DP expansion, and examination of the DN subpopulations revealed a subtle block at the DN3-DN4 transition (Fischer et al., 2005). Moreover transgenic over-expression of the TCF target gene Egr-1 in $RAG^{-/-}$ animals results in cells bypassing the β-selection checkpoint and developing into ISPs (Miyazaki, 1997), while

mice deficient in both Egr-1 and Egr-3 have been shown to have a block at DN3 (Carter et al., 2007). Interestingly, the use of a constitutively active form of SAP-1 is capable of inducing immature single positive (ISP) development on a $RAG^{-/-}$ background (Costello unpublished observations). Also the results of the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ bone marrow chimera experiments suggest that TCF activity is required during early thymocyte development, although the nature of this requirement is unclear. It has been observed that SAP-$1^{-/-}$ Elk-$1^{-/-}$ $CD4^+$ T cells are hypoproliferative both *in vitro* and *in vivo* (Costello unpublished observations). Thus it may be that SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ thymocytes have a defect in proliferation which may impact on the proliferative expansion during the DN-DP transition. Alternatively, SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ thymocytes may be unable to compete for factors required for migration or thymocyte survival. Defects in migration have been reported in $Net^{\delta/\delta}$ mice (Buchwalter et al., 2005; Zheng et al., 2003). Thus experiments examining the migration and proliferation of SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ cells may provide insights into a possible role for the TCFs in early thymocyte development.

However the requirement for TCFs during early thymocyte development only becomes apparent in conditions where they are competing with WT thymocytes. This suggests that the developmental signals can be interpreted correctly by SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ thymocytes. This in turn suggests that Egr activity is normal. Egr-1 activity has been shown to be enhanced by NAB-2 in T cell clones (Collins et al., 2006). Furthermore NAB-2 expression was induced by TCR activation, partially in an NFAT dependent manner. Thus it is possible that interactions of NAB-2 and Egr-1 are sufficient to compensate for the loss of the TCFs.

7.5 Regulatory T cell development

7.5.1 TCFs and T_{reg} development

Since the identification of a regulatory T cell population within the $CD4^+CD25^+$ T cell population by Sakaguchi and co-workers (Sakaguchi et al., 1995) there has been much interest in regulatory T cells. Whilst the signalling pathways responsible for positive and negative selection have been extensively studied, the requirements for T_{reg}

development are less well understood. It has generally been assumed that T_{regs} arise following positive selection (Modigliani et al., 1996; reviewed in Sakaguchi, 2004). However, T_{regs} have a different TCR repertoire to conventional SP thymocytes (Pacholczyk et al., 2006). T_{regs} require interaction between the TCR and MHC for their selection, whilst TCR transgenic experiments have suggest that this is a high affinity interaction (Bensinger et al., 2001; Hori et al., 2002; Itoh et al., 1999; (Jordan et al., 2001). However how TCR signals lead to T_{reg} development is unclear.

Through the use of TCF deficient mice it was shown that that T_{reg} development is independent of the TCF SAP-1. It is also possible that T_{reg} development is also Elk-1 and Net independent. Disruption of these genes individually did not result in any defects in T_{reg} development. Furthermore deletion of Elk-1 on a SAP-$1^{-/-}$ background, whilst exacerbating the positive selection defect observed in SAP-$1^{-/-}$ mice, did not result in a change in the absolute number of T_{regs} in these animals, with the numbers remaining equivalent to that observed in WT animals. The thymic cellularity was increased in SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals so it may have been expected that if T_{reg} development was unaffected then the absolute numbers of T_{regs} would have increased. However several studies have reported that despite an increased proportion of T_{regs}, and perceived increased entry of precursors into the T_{reg} lineage, the absolute numbers did not increase significantly (Jordan et al., 2001; Apostolou et al., 2002; van Santen et al., 2004). These studies have contributed to the "two signal" model of T_{reg} development whereby the second signal is present in limiting quantities (reviewed in Liston and Rudensky, 2007). Therefore it may be that in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals the increased thymic cellularity would not necessarily result in an increased absolute numbers of T_{regs} even if their development were unaffected. Thus at present there is no conclusive data to indicate that T_{reg} development is defective in the SAP$^{-/-}$ Elk-$1^{-/-}$ animals however, further experiments are need to conclusively determine if T_{reg} development is independent of both SAP-1 and Elk-1 or if a threshold of TCF activity is required. One experiment which may provide insight into this matter would be to examine SAP-$1^{-/-}$ Elk-$1^{-/-}$ T_{reg} development in a competitive environment such as the mixed bone marrow chimera experiments. Another possibility is that if the "two signal" model is correct, identification of the limiting factor may allow examination of this factor in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals and give an indication as to whether the absolute numbers of T_{regs} should have increased.

A further consideration with the SAP-$1^{-/-}$ Elk-$1^{-/-}$ T_{reg} data is the increased proportion of CD103^{+} T_{regs} in the thymi of these animals. This increase is also present in

the periphery, although it is unclear why this increase has occurred. Whilst the importance of this increase is not apparent, the CD103 marker is thought to be a marker for activated/memory T_{regs}. Therefore the increased proportion in the thymus may indicate some recirculation of T_{regs} from the periphery to the thymus. This possibility needs to be explored further so that the calculation of absolute thymic numbers of T_{regs} is not falsely inflated by the presence of peripheral T_{regs}. One possibility would be to examine T_{reg} development in fetal thymic organ cultures and if this method faithfully recapitulated T_{reg} development, then analysis of SAP-$1^{-/-}$ Elk-$1^{-/-}$ thymocytes in this system may provide further insight into whether T_{reg} development is defective or not in these animals. One study has shown that they Foxp3 cells can develop in FTOC when culture with thymic stromal derived lymphopoietin (TSLP) (Jiang et al., 2006), and so this may be something which can be pursued by other members of the transcription laboratory. The increase in $CD103^{+}$ T_{regs} was not observed in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals and it is not clear at present why this is the case.

Deletion of the Net TCF did not appear to affect positive selection, either alone or in combination with SAP-1 or SAP-1 Elk-1 deficiency. T_{regs} were clearly detectable in SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ mice, at a proportion similar to that seen in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ animals. These data show that T_{reg} selection is not mediated by specific TCF proteins, and suggests that the signalling requirements for T_{reg} development are different to the requirements for positive selection.

The slight reduction in T_{reg} numbers in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals could be viewed as indicating that a low threshold level of TCF activity is required during regulatory T cell development. One consideration with regards to these data concerns whether the Netδ mutation represents a true null mutation. The $Net^{\delta/\delta}$ mice were generated by a deletion of a region containing the initiation codon and part of the DNA binding domain (Ayadi et al., 2001). The remaining mRNA sequence contained an alternative translation start site that allowed production of an N-terminally truncated shorter Net protein (Netδ). The authors proposed that Netδ is at least a hypomorphic mutant (Ayadi et al., 2001), which would potentially explain the lack of genetic interaction with the other TCF proteins in this system. Against this, it is important to note that this protein does not function in reporter assays containing its target binding site (Ayadi et al., 2001), and removal of the Ets binding domain in other TCFs abolishes binding and complex formation with SRF both *in vitro* and *in vivo* (Dalton and Treisman, 1992;Price et al., 1995). Furthermore, it is unlikely that this truncated protein acts as a dominant negative mutant as $Net^{\delta/-}$ do not display any phenotype (Ayadi et al.,

2001). For these reasons we favour the view that the Netδ mutation is a null mutation, although it is not possible to formally exclude that it acts as a dominant negative or hypomorph mutant.

A further consideration with regard to analysing absolute numbers of T_{regs} in these reconstituted animals is that the irradiation reconstitution approach did not faithfully recapitulate the increased thymic cellularity observed in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ donor animals. The SAP-$1^{-/-}$ Elk-$1^{-/-}$ $CD4^+$ peripheral T cells have been demonstrated to have a defect in proliferation (P.C and R.T. unpublished observations) thus it may be that this is also the case in the thymus and as such reconstitutions using cells from these animals may take longer to reach a steady state of thymic cellularity. It is possible that this is also true for the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ cells. Thus it is possible that the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ reconstituted animals should have shown an increased thymic cellularity (if it had been possible to generate adult SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ donors) and thereby account for the slight reduction in numbers of regulatory T cells.

However, the reduced absolute numbers of T_{regs} in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals may in fact represent a requirement for the TCFs in T_{reg} development. Although another possibility was that this reduction could be due to a reduction in the presence of a limiting factor as proposed by the two-signal model rather than a cell intrinsic requirement for TCF activity. This possibility was addressed by mixed bone marrow chimera experiments. These experiments revealed that the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ cells did not compete efficiently with WT cells however, this prevented comparison of absolute numbers of T_{regs} derived from these two sets of cells. The proportion of regulatory T cells derived from SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ cells in the mixed chimera was significantly enhanced compared with animals reconstituted with SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ cells alone whilst the proportion of $CD4^+$ SP T cells was consistent between the two experiments. These data suggest that T_{reg} development from SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ derived cells occurs more efficiently in the mixed chimera experiments, although this is not conclusive without data regarding absolute numbers. However in one animal where a 1:1 ratio of SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ to WT derived cells did occur, the absolute numbers of T_{regs} was equivalent. Thus by examining more animals with a 1:1 contribution in the mixed bone marrow chimeras it may be possible to determine whether under competitive conditions, SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ T_{reg} development is defective or alternatively is a TCF independent process. Another consideration with these experiments is that it is not possible to distinguish between CD45.1 cells derived from

the injected bone marrow and recovering host cells, therefore these experiments should be performed in a manner which would allow for this discrimination, perhaps by using irradiated $RAG^{-/-}$ mice.

It is clear that while the TCFs play a critical role in thymocyte positive selection, the TCF SAP-1 is not required for T_{reg} development and at present it is not possible to conclusively identify a role for the TCFs in T_{reg} development. Thus regulatory T cell development has distinct requirements from positive selection. Whilst one possibility is that the TCFs are simply not required for T_{reg} development or that a lower threshold of activity is required, another possibility is that the TCFs are not required for positive selection as such but are required post-positive selection for efficient proliferation. This would also result in an apparent lack of requirement for the TCFs in T_{reg} development as T_{regs} are thought to undergo substantially fewer rounds of proliferation compared with conventional SP thymocytes and typically present an anergic phenotype. The TCFs have been proposed to be important for proliferation of peripheral T cells. In particular it has been shown that SAP-$1^{-/-}$ Elk-$1^{-/-}$ $CD4^+$ peripheral T cells are hypoproliferative, however the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $CD8^+$ peripheral T cells are hyperproliferative both *in vitro* and *in vivo* (P.C. and R.T. unpublished observations). Whilst the proliferation of SAP-$1^{-/-}$ Elk-$1^{-/-}$ thymocytes has not been examined, further experiments are required to explore this possible explanation as to the differential requirement of TCF signalling between positive selection and T_{reg} development.

7.5.2 SRF is required for T_{reg} development

Deletion of the TCF transcription partner SRF revealed an almost complete block in the generation of SP thymocytes and regulatory T cells. This contrasts with the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals, raising the possibility that positive selection involves both TCF-dependent and TCF-independent components. Other members of the lab are exploring this possibility further by analysing SRF mutants that are capable of interacting with TCF but not other partners. Understanding the difference between the SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals and the SRF-depleted animals may provide valuable insights into the requirements for T_{reg} development.

In addition to the TCFs, SRF interacts with the Myocardin Related Transcription Factors (MRFTs) which are responsible for transcription regulation of a number of muscle-specific genes including actin and smooth muscle actin in response to Rho

signalling (Miralles et al., 2003; Gineitis and Treisman, 2001). Deletion of SRF will also disrupt this signalling pathway and indeed it has previously been reported that conditional deletion of SRF in the heart results in lethal cardiac defects (Parlakian et al., 2004). The regulation of the cytoskeleton has been shown to be important for synapse formation between lymphoid cells. Synapse formation has been observed in response to MHC-TCR interactions. Since MHC-TCR interaction are required for positive and negative selection, one might postulate that if thymocytes are defective in forming conjugates then current understanding suggests that these processes would not be able to occur. Moreover interactions between TCR and MHC have also been shown to be required for T_{reg} development and as such a failure to form conjugates would also affect T_{reg} numbers. However studies in the laboratory suggest that SRF deleted DP thymocytes can form conjugates, although the efficacy of these conjugates to signal has not been assessed (P. Costello personal communication).

Another possibility is that SRF is required to produce a survival signal for T_{regs}, possibly mediated through Rho-MRTF-SRF pathway. Although SRF has been shown to interact with other transcriptional partners (Philippar et al., 2004; Chen and Schwartz, 1996; Belaguli et al., 2000), Rho has previously been demonstrated to be important for thymocyte development (Cleverley et al., 1999). Future experiments may incorporate gene expression studies to identify a potential survival factor that may be differentially regulated between $SRF^{f/f}$ CD2-Cre^{+} and SAP-$1^{-/-}$ Elk-$1^{-/-}$ $Net^{\delta/\delta}$ animals. Interestingly Bcl2 has been shown to be regulated by SRF and may a starting point to identify any potential survival mechanism (Schratt et al., 2004).

A further approach to investigate the basis for the defect in SRF-null thymocytes is to use retroviral expression to examine SRF mutants in $SRF^{f/f}$CD2-Cre^{+} bone marrow reconstitutions. This will allow the examination of whether differential cofactor binding is important during thymocyte development, possibly providing insights into the TCF-independent process that occurs during positive selection. Furthermore these mutants may also provide insights into the requirements for regulatory T cell development, as it is possible that some mutants will rescue positive selection but not T_{reg} development.

7.5.3 Ras-ERK signalling in T_{reg} development

The TCF data demonstrated that the signalling requirements for T_{reg} development and positive selection were different; therefore the role of ERK signalling

was examined in this process. $ERK1^{-/-}$ mice were reported to have a 50% reduction in the proportions of SP thymocytes (Pages et al., 1999) however, we were not able to reproduce this. In our hands $ERK1^{-/-}$ mice displayed a maximum of 20% reduction in SP thymocyte proportion and no difference was observed in the proportion of $CD4^+CD25^+$ T cells. If T_{reg} development had been unaffected in these mice, it may have been predicted that the proportion of $CD4^+CD25^+$ T cells would increase as demonstrated for the TCF deficient mice. Whilst these data may indicate a role for ERK signalling in T_{reg} development, it may be that a 20% increase would not be detected.

Further insight into the role of ERK signalling in T_{reg} development can be gleaned from the analysis of the conditional $ERK2^{-/-}$ mouse which demonstrated that ERK2 has the greater role in positive selection, and that there is redundancy between ERK1 and ERK2 (Fischer et al., 2005). In the mice deficient for both ERK1 and ERK2, gating on $TCR^{hi}CD69^+$ demonstrated that the proportion of mature thymocytes was reduced to 0.1% from 15% in WT animals. In experiments with SAP-1 reconstituted animals regulatory T cells were examined for TCR^{hi} expression and the numbers of $TCR^{hi}CD4^+Foxp3^+$ T cells were found to be comparable to the numbers of $CD4^+Foxp3^+$ T cells. As T_{regs} represent approximately 5% of the mature $CD4^+$ population as a proportion of total WT thymocytes they are likely to represent approximately 0.5%. Therefore, although it was not specifically addressed, if T_{reg} development had been normal in these mice, the proportion of mature thymocytes would have been expected to be approximately 5 times higher.

My examination of mice expressing a dominant negative Raf transgene revealed clear defects in T_{reg} development and therefore implicated the Ras-ERK signalling pathway in this process. One potential explanation for the reduction in regulatory T cell numbers in DN Raf mice is defective proliferation (O'Shea et al., 1996). This would appear unlikely as it has been proposed that T_{regs} may derive from a population of thymocytes which undergo few rounds of proliferation (Pennington et al., 2006) and as such any proliferation defect would be unlikely to have much of an effect. Furthermore these cells are usually anergic and so unlikely to be affected by defects in proliferation. However under certain conditions, T_{regs} have been shown to expand *in vivo*, therefore this possibility requires further examination.

Whilst it is probable that the reduced numbers of regulatory T cells in mice expressing the DN Raf transgene is as a result of a requirement of ERK signalling in T_{reg} development, it remains possible that the DN Raf transgene affects other signalling pathways than just the Ras-Raf-ERK pathway. Raf-1 has been shown to be involved in

regulation of apoptotic pathways through its inhibitory interactions with the pro-apoptotic kinases MST2 and ASK1 (Chen et al., 2001; O'Neill et al., 2004). However the DN Raf product still contains the N- terminal domain which is required for these interactions, thus if the DN Raf was likely to have any affect on these pathways, most likely it would increase the inhibition of apoptosis and thus lead to an increase in cell numbers. Evidence is still emerging about the role of scaffold proteins in the Ras-ERK pathway, in particular several have been identified which interact or act on RAF such as KSR and CNK (Kortum and Lewis, 2004; Ziogas et al., 2005). CNK has also been shown to regulate the RhoGTPase transcriptional activities (Jaffe et al., 2004), thus it is possible that the DN Raf transgene has affects on other pathways through interactions with scaffold proteins, the consequences of which have not been fully examined.

To further investigate the role of ERK signalling in T_{reg} development the technique called 'peripheral conversion' was employed in which naïve T cells can be converted in Foxp3 expressing cells that have suppressive activities. These experiments demonstrated that it was possible to induce Foxp3 expression in naïve T cells. Furthermore this could be blocked by the MEK inhibitor UO126, thereby implicating a role for ERK in this process, and a similar result was obtained when preliminary experiments were performed with DN Raf cells (JW unpublished observations). Although it could be argued that UO126 could be acting to block the expansion of contaminating Foxp3 cells rather than the *de novo* expression of Foxp3, this would appear unlikely since TGFβ does not induce the proliferation of T_{regs} (Fantini et al., 2004). Further experiments where the additional CD45RB marker is employed to reduce the number of $Foxp3^+$ T cell at the start of the experiment, coupled with BrdU analysis to monitor proliferation should clarify this point.

The involvement of the Ras-ERK signalling pathway in both regulatory T cell development and positive selection presented here suggests that ERK may mark a branch point between the process of positive selection mediated through the TCFs and the development of regulatory T cells mediated by at present an unknown target of Ras-ERK signalling. It is important to remember that the DN Raf animals have a reduced ability to signal through ERK and not a complete block in ERK signalling. Thus it may be that a complete block or a more substantial block of ERK signalling would result in a complete block in T_{reg} development. Since deletion of ERK1 and ERK2 also affects positive selection, more subtle experiments might be needed to directly address this issue. However this would provide further insight into the relative requirements for ERK signalling and TCF activity.

The DN Raf data and peripheral conversion data would also suggest an involvement of ERK signalling in the induction of Foxp3 expression. Studies of the Foxp3 promoter may provide further insights into the signalling requirements for T_{reg} development. Little is known about the regulation of Foxp3 expression. The peripheral conversion assay has shown that TGFβ can induce Foxp3 expression. Furthermore the results present here demonstrate a role for ERK in TGFβ induced expression. Recently it has been proposed that Foxp3 is not the initiator of the regulatory T cell lineage (Gavin et al., 2007; Lin et al., 2007). If this is the case then it is possible that under certain conditions, TGFβ signals bypass the initiation factor to directly up-regulate Foxp3. Thus providing a possible explanation for the differences between signals required for peripheral conversion and thymic development.

7.5.4 A question of affinity?

A simple interpretation of the findings presented here is that the signalling pathways downstream of the TCR that lead to expression of Foxp3 and commitment to the T_{reg} lineage are different from those involved in positive selection, even though both processes are dependent on TCR-MHC-peptide interactions. It remains possible however, that as the affinity of the interaction increases the dependence of positive selection on ERK-SAP-1 decreases such that high affinity TCRs, such as those inducing T_{reg} selection, no longer require ERK-SAP-1 signalling to escape death by neglect. According to this view, inactivation of ERK-SAP-1 signalling would differentially affect selection according to the affinity of the TCR-peptide interaction, resulting in a loss of conventional $CD4^+$ T cells but not T_{regs}. Interestingly so far a role for the TCFs in negative selection, another process dependent on high affinity interactions, has not been demonstrated.

One implication of this hypothesis is that the repertoire of the conventional T cells which are selected in SAP-$1^{-/-}$ animals would be skewed towards higher affinity TCRs, which would in turn increase the chances of developing autoimmunity. Interestingly there are indications SAP-$1^{-/-}$ animals may suffer from an autoimmune response. Initial characterisation of the SAP-$1^{-/-}$ phenotype included the development of Castleman's disease like symptoms. Analysis of the mice showed the presence of DNA antibodies, which can be an indication of autoimmunity (R. Nicolas / Y. Watanabe, unpublished observations). However since the line has been fully backcrossed onto the

BL6 background these observations of Castleman's disease have not been repeated. During experiments to recapitulate the Castleman's phenotype under controlled conditions, age-matched WT and SAP-$1^{-/-}$ mice were examined and it was found that DNA antibodies could be detected at about 1 year of age in SAP-$1^{-/-}$ mice but not WT animals (J.W. unpublished observations), again suggesting that SAP-$1^{-/-}$ animals might be more susceptible to autoimmunity. Different strains of mice have differing susceptibility to develop autoimmunity with BL6 being one of the least susceptible. For instant the lpr mutation of Fas on the MRL background results in autoimmune disease however, on the BL6 or C3H/HeJ background no autoimmune disease was observed (Yajima et al., 2003). Thus the mixed background of the initial SAP-$1^{-/-}$ mice might explain the more severe symptoms observed in these animals and the apparent lack of symptoms in the fully backcrossed mice.

Examination of the affinities which direct either negative or positive selection have proposed that there is a very sharp border defining these two events, without a intermediate area where T_{regs} might be selected (Daniels et al., 2006). If it was shown that the loss of SAP-1 skewed the repertoire towards high affinity receptors, thereby explaining the normal development of T_{regs} in the SAP-$1^{-/-}$ animals, it may provide evidence that T_{regs} are rescued from negative selection.

7.6 Regulatory T cell function

7.6.1 Signalling in Regulatory T cells

Regulatory T cell function requires activation through the TCR (Thornton and Shevach, 1998; Takahashi et al., 1998). Examination of signalling downstream of the TCR revealed that although the Ras-ERK pathway could be activated, the induction was lower in T_{regs}. This confirmed recent reports that T_{regs} have distinct responses to TCR signalling (Hickman et al., 2006; Tsang et al., 2006) when compared with conventional $CD4^+$ T cells. Traditional activation of T cells with αCD3 and secondary signals, either from αCD28 or APCs, fails to induce a proliferative response in T_{regs} however they have been shown to proliferate *in vitro* in response to IL-2 and proliferate rapidly *in vivo* (Thornton and Shevach, 1998; Takahashi et al., 1998; Walker et al., 2003). These

results suggest that T_{regs} are not globally unresponsive to TCR stimulation but have specific requirements which are distinct from conventional $CD4^+$ T cells furthermore, these results may help to explain the anergic phenotype *in vitro*.

7.6.2 TCFs and regulatory T cell function

Examination of TCF deficient regulatory T cells in *in vitro* and *in vivo* models of suppression demonstrated that T_{regs} do not require the TCFs SAP-1 and/or Elk-1 for their function. Initial experiments in collaboration with F. Powrie suggested a subtle defect in SAP-$1^{-/-}$ T_{regs} when added at a 1:4 ratio (T_{reg}:Effector), this was not observed at a 1:2 ratio. Furthermore preliminary experiments where SAP-$1^{-/-}$ Elk-$1^{-/-}$ T_{regs} were added at a 1:4 ratio showed there was no obvious defect in suppressive capability (JW unpublished observations). If TCFs had been required for T_{reg} function *in vivo* it would have been expected to be more apparent in the SAP-$1^{-/-}$ Elk-$1^{-/-}$ T_{regs}. However these results are only preliminary and must be interpreted with caution due to the lower than expected levels of colitis in the control group in these initial experiments. Interestingly, other *in vivo* models have been shown to have subtly different requirements for *in vivo* suppression by T_{regs}, for instance suppression of autoimmune thyroiditis requires IL-4 not IL-10 as in the colitis model (Seddon and Mason, 1999; Asseman et al., 1999). Thus alternative *in vivo* suppression models may provide evidence for a role for TCFs in T_{reg} function. The data presented indicates that T_{reg} function is independent of the TCFs SAP-1 and Elk-1 at least in the models used in this thesis, although T_{reg} function *in vivo* requires further examination.

7.6.3 ERK signalling and T_{reg} function

In vitro suppression assays demonstrated that DN Raf T_{regs} are fully functional, indicating that Ras-ERK signalling is not required for T_{reg} function. Unfortunately a limited supply of animals barred the possibility of assessing these cells in the *in vivo* model of IBD, so it is not possible to rule out a role for ERK signalling in *in vivo* suppression.

Attempts to further assess the role of ERK signalling in T_{reg} function involved examining the requirements for pre-activation of T_{regs}. IL-2 has been shown to be important during pre-activation of regulatory T cells and the increased function demonstrated in these cells (Thornton et al., 2004). Pre-activation of regulatory T cells in the presence of the MEK inhibitor UO126 blocked the increase in suppressive function of the "pre-activated" T_{regs}, although they were still capable of suppressing proliferation, thus indicating a possible role for ERK during this pre-activation stage. It is unclear how the pre-activation of T_{regs} *in vitro* relates to their *in vivo* function. There is increasing evidence that T_{regs} are present at sites of infection and inflammation (Belkaid et al., 2002) and so it may be that once the immune response has reached a critical level, with localised high levels of IL-2 production, that this is the signal for T_{regs} to become fully activated tipping the balance between effectors and T_{regs} sufficiently to dampen down the response and so prevent tissue damage and autoimmunity.

7.7 Future Directions

It is possible DN Raf T_{regs} are not functional *in vivo*. Furthermore suppression *in vivo* may have stricter activation requirements that an *in vitro* assay. In order to investigate the relevance of ERK signalling in the activation of T_{regs}, the function of DN Raf T_{regs} could be examined in the *in vivo* model of colitis.

In addition to the possible experiments outlined above, directed at further understanding the role of ERK signalling in T_{reg} development and function, data presented here have also provided interesting observations which could lead to other areas of investigation which I detail below.

7.7.1 Examining the affinity repertoire of TCF deficient animals

One hypothesis regarding the data presented here is that the loss of SAP-1 skews the TCR towards high affinity receptors. To directly assess the impact of SAP-1 on high and low affinity selection we have set up a collaboration with Dr. Caton. Dr Caton's group have previously generated transgenic mice expressing TCRs which recognise the HA peptide with approximately 100 fold difference in affinity for the HA peptide

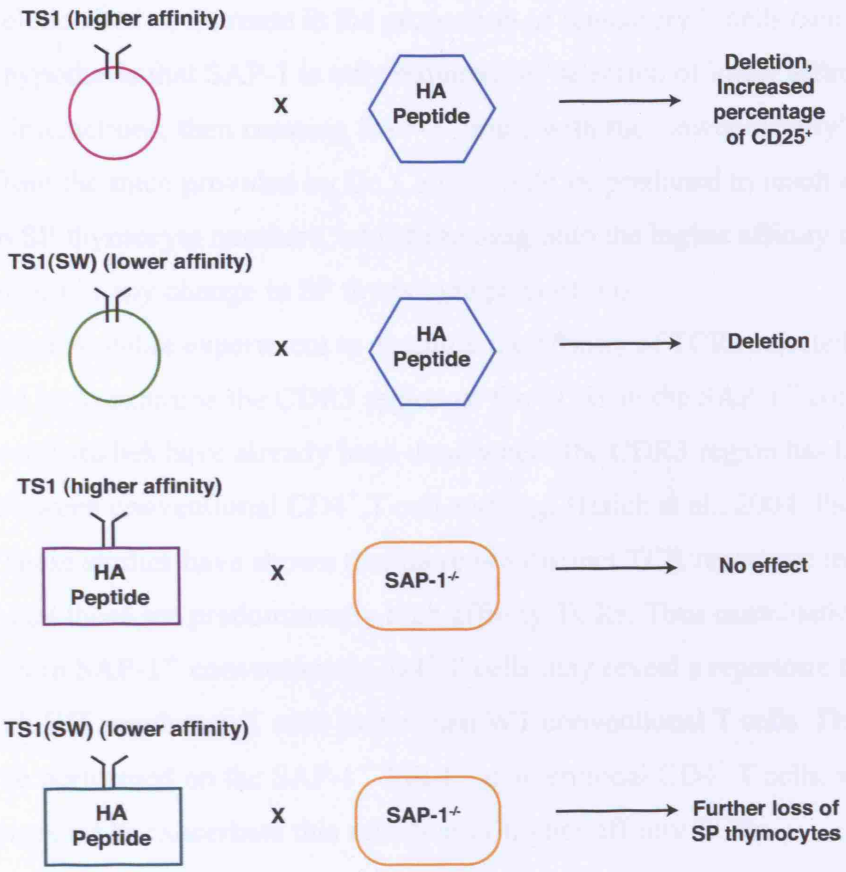


Figure 7.1. Transgenic experiments to investigate if loss of SAP-1 skews the TCR repertoire. As demonstrated by Dr. Caton's group, crossing mice expressing the TS1 TCR which has a high affinity for the HA peptide, onto mice expressing that peptide, results in some deletion but a substantial increase in the proportion of $CD4^+CD25^+$ T cells demonstrating that T_{regs} are selected on a high affinity interaction. However crossing mice expressing the lower affinity TCR TS1(SW) only results in deletion. I postulate that if loss of SAP-1 skews the repertoire towards high affintiy TCRs, crossing $SAP\text{-}1^{-/-}$ mice onto the higher affinity TCRxHA cross will have no further effect whilst crossing onto the lower affintiy TCRxHA cross will result in a further reduction in the numbers of SP thymocytes.

(Jordan et al., 2001). Crossing of the higher affinity TCR to mice expressing the HA peptide resulted in a high affinity cross and therefore deletion, but a striking increase in the proportion of $CD4^+CD25^+$ T cells was also observed. On the other hand, crossing of the lower affinity TCR to mice expressing the HA peptide resulted in a lower affinity cross and deletion but no increase in the proportion of regulatory T cells (see Figure 7.1). If the hypothesis that SAP-1 is only required for selection of lower affinity TCR:MHC interactions, then crossing SAP-$1^{-/-}$ mice with the "lower affinity" cross generated from the mice provided by Dr. Caton would be predicted to result in a further reduction in SP thymocyte numbers, whilst crossing onto the higher affinity cross would not result in any change in SP thymocyte proportions.

Another possible experiment to examine the affinity of TCRs selected in SAP-$1^{-/-}$ mice would be to examine the CDR3 region of the TCRs in the SAP-$1^{-/-}$ conventional T cells. Several studies have already been done where the CDR3 region has been compared between conventional $CD4^+$ T cell and T_{regs} (Hsieh et al., 2004; Pacholczyk et al., 2006). These studies have shown that there is a distinct TCR repertoire in regulatory T cells and that these are predominantly high affinity TCRs. Thus examination of the CDR3 region in SAP-$1^{-/-}$ conventional $CD4^+$ T cells may reveal a repertoire that overlaps with WT regulatory T cells rather than WT conventional T cells. This analysis could also be performed on the SAP-$1^{-/-}$ Elk-$1^{-/-}$ conventional $CD4^+$ T cells, which would be expected to exacerbate this selection of higher affinity TCRs.

If the loss of SAP-1 does skew the TCR affinity then it might be expected that SAP-$1^{-/-}$ mice would be more prone to autoimmunity. As previously stated it is possible to detect anti-DNA antibodies in the SAP-$1^{-/-}$ mice. To further examine the possibility that SAP-$1^{-/-}$ mice are more susceptible to autoimmunity experiments could be performed where SAP-$1^{-/-}$ mice are immunologically challenged with a crude preparation of yeast cell wall. This was performed in mice with a defect in Zap-70 which in SPF conditions do not develop arthritis however, injection of Zymosan (yeast cell wall extract) was sufficient to trigger development of autoimmune arthritis (Yoshitomi et al., 2005; reviewed in Sakaguchi et al., 2006). Thus it may be that autoimmune disease occurs when SAP-$1^{-/-}$ animals are immunologically challenged. Furthermore, if the SAP-$1^{-/-}$ mice have a higher susceptibility to autoimmune disease then this may be exacerbated in SAP-$1^{-/-}$ Elk-$1^{-/-}$ mice, therefore it would be interesting to examine SAP-$1^{-/-}$ Elk-$1^{-/-}$ mice with age-matched controls and see if DNA antibodies can be detected. If it was possible to demonstrate an increased susceptibility to autoimmunity in TCF deficient mice, it is feasible to suggest that this may be as a result

of T cells with high affinity TCRs being selected, although this would not be the only possible explanation.

7.7.2 Function of conventional $CD4^+$ T cells

Th1/Th2 differentiation has been reported to be influenced by TCR affinity (Boyton and Altmann, 2002), with high affinity interactions promoting Th1 differentiation. Other models of Th1/Th2 differentiation have implicated ERK as important for the Th2 cytokine IL-4 (Jorritsma et al., 2003). However initial experiments to look at *in vitro* differentiation of SAP-$1^{-/-}$ naïve T cells failed to show any bias towards either Th1 or Th2 differentiation, instead an increased production of cytokines was observed under all culture conditions. These results need repeating to confirm that there is no bias towards either Th1 or Th2. Examination of T-bet and GATA-3 expression (two genes critical for correct T helper differentiation) may also provide insights into the early stages of T helper differentiation and whether the initial commitment to either lineage is SAP-1 independent.

A further area of investigation should examine the recently identified Th17 population of effector cells. These cells are now highly implicated in inflammatory and autoimmune responses. These cells have their own characteristic cytokine profile expressing high levels of IL-6 and require IL-6 and TGFβ for their differentiation (Weaver et al., 2006). The original phenotype of the SAP-$1^{-/-}$ animal was the development of neck lumps, primarily made up of plasma cells, and high levels of IL-6 being detected. Whilst this could be as a result of the development of the neck lump and being produced by the plasma cells, it is also possible that it is as a result of the presence of Th17 cells.

7.8 Conclusions

In this thesis I have shown that whilst the TCFs are important for positive selection of conventional T cells, T_{reg} development has a different requirement for TCF activity. Furthermore TCF deficient T_{regs} are functional in the models of suppression that have been used here. Interestingly the TCF transcriptional partner SRF is required for T_{reg} development possibly indicating a role for Rho signalling in T_{reg} development. These

results also indicated that there is an SRF-dependent TCF-independent process during positive selection. Finally it was shown that the Ras-ERK signalling pathway is involved in T_{reg} development as well as positive selection. Taken together these data suggest that the signals required for T_{reg} development are different to the requirements for positive selection and that there may be a branch point in these signals at the level of ERK activation and the subsequent activation of ERK targets.

8 Appendix

Results presented here are all from preliminary experiments and require further experimentation before firm conclusions can be drawn from them. However these results may provide useful for members of the transcription laboratory who may continue with this line of investigation and thus are recorded here.

8.1 Are SAP-1$^{-/-}$ Elk-1$^{-/-}$ T_{regs} functional *in vivo*?

8.1.1 Introduction

SAP-1$^{-/-}$ and SAP-1$^{-/-}$ Elk-1$^{-/-}$ T_{regs} have been shown to be functional *in vitro* and the SAP-1$^{-/-}$ T_{regs} have been shown to be functional in an *in vivo* model of IBD (see section 5.7). However when the SAP-1$^{-/-}$ T_{regs} were added at a 1:4 ratio it was unclear whether they were as efficient at WT T_{regs}. Whilst this could be explored further using other models of *in vivo* suppression, another possibility was that any defect would be exacerbated in the SAP-1$^{-/-}$ Elk-1$^{-/-}$ T_{regs}. Therefore the SAP-1$^{-/-}$ Elk-1$^{-/-}$ T_{regs} were examined in the *in vivo* model of IBD.

8.1.2 Results

Since the *in vivo* model of IBD hinted that SAP-1$^{-/-}$ T_{regs} might be subtly defective, SAP-1$^{-/-}$ Elk-1$^{-/-}$ T_{regs} were assessed in this model in order to test whether further reduction of TCF activity would accentuate any defect. Due to reduced fertility in the SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals it was difficult to obtain sufficient regulatory T cells from the these animals, therefore the experiment was set up using a 1 T_{reg}:4 Effector T cell ratio and cells were isolated from both spleens and lymph nodes. The progression of the disease was once again assessed by measuring the weights of the individual mice.

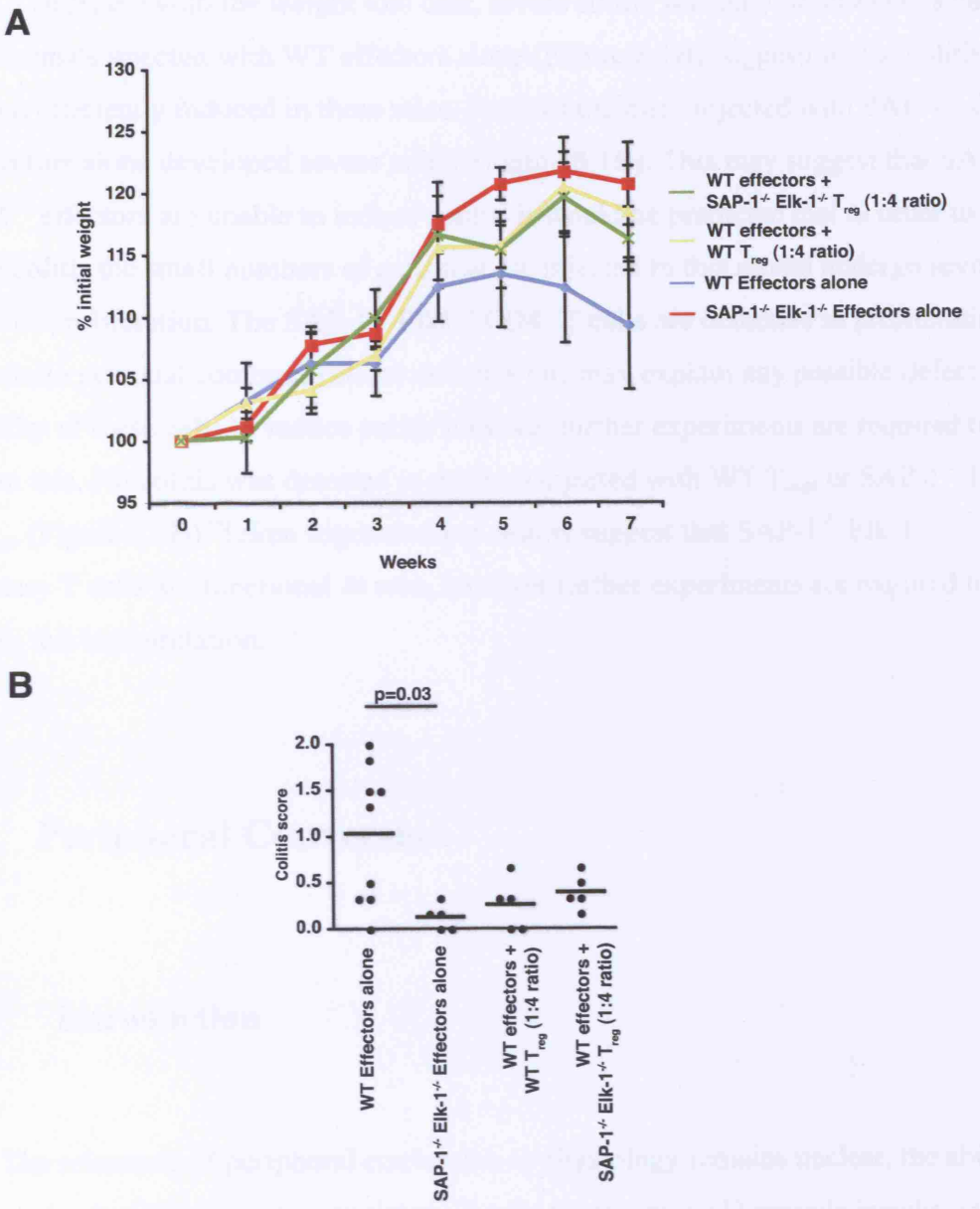


Figure 8.1. Examination of SAP-1$^{-/-}$ Elk-1$^{-/-}$ T_{reg} function *in vivo*. The colitis model of Inflamatory Bowel Disease (IBD) was used to assess function of T_{regs} *in vivo*. Animals were injected with naive effector T cells either alone or with T_{regs} from WT or SAP-1$^{-/-}$ Elk-1$^{-/-}$ animals. T_{regs} added at a 1 T_{reg}:4 Effector T cells ratio. **A.** Disease progression was monitored by measuring the weight of mice. Once mice lost 20% of their weight, as directed by our Ethics Committee guidelines, mice were sacrificed and the experiment terminated. **B.** Histological analysis was performed to generate scores of colitis severity.

Very little weight loss was observed in any of the groups during the course of the experiment (Figure 8.1A) and therefore the experiment was arbitrarily terminated at 7 weeks. Consistent with the weight loss data, severe colitis was only observed in a half of the animals injected with WT effectors alone (Figure 8.1B) suggesting that colitis has not been efficiently induced in these mice. None of the mice injected with SAP-$1^{-/-}$ Elk-$1^{-/-}$ effectors alone developed severe colitis (Figure 8.1B). This may suggest that SAP-$1^{-/-}$ Elk-$1^{-/-}$ effectors are unable to induce colitis. It would be predicted that in order to induce colitis the small numbers of cells that are injected in this model undergo several rounds of proliferation. The SAP-$1^{-/-}$ Elk$1^{-/-}$ $CD4^+$ T cells are defective in proliferation (P. Costello personal communication) and thus this may explain any possible defect in the ability of these cells to induce colitis however further experiments are required to confirm this. No colitis was detected in mice co-injected with WT T_{regs} or SAP-$1^{-/-}$ Elk-$1^{-/-}$ T_{regs} (Figure 8.1B). Taken together these results suggest that SAP-$1^{-/-}$ Elk-$1^{-/-}$ regulatory T cells are functional *in vivo*, however further experiments are required to confirm this interpretation.

8.2 Peripheral Conversion

8.2.1 Introduction

The relevance of peripheral conversion to physiology remains unclear, the ability to convert naïve effectors into regulatory T cells *in vitro* may (1) provide insights into the development of naturally occurring regulatory T cells; and (2) may be of clinical relevance. It was possible to show that the process of peripheral conversion required ERK signalling (See section 6.9.1.2). To further examine the requirements of the peripheral conversion process and assess whether these were similar to those for thymic development of regulatory T cells, DN Raf and SAP-$1^{-/-}$ naïve T cells were examined for their ability to undergo peripheral conversion.

8.2.2 Results

$CD4^+CD25^-$ T cells were isolated from WT, SAP-$1^{-/-}$ and DN Raf transgenic mice. The cells were cultured in αCD3, IL-2, APCs and TGFβ for 3 days before analysis of Foxp3 expression was performed. Basal levels of Foxp3 expression were similar between WT and SAP-$1^{-/-}$ $CD4^+CD25^-$ T cells, however the level of Foxp3 expression was slightly elevated in DN Raf $CD4^+CD25^-$ T cells (Figure 8.2 top row). This increased Foxp3 expression in the DN Raf cells may indicate some level of contamination with T_{regs}.

Upon activation in the presence of TGFβ the proportion of cells expressing Foxp3 was substantially increased in WT cultures (Figure 8.2 middle row, left panel). An increase in cells expressing Foxp3 was also observed in the SAP-$1^{-/-}$ cultures however, this was lower than that observed in WT cultures (Figure 8.2 middle row middle panel). Examination of the conversion of DN Raf cells also displayed a reduced induction of Foxp3 (Figure 8.2 middle row left panel).

Addition of the MEK inhibitor UO126 to all the cultures, including DN Raf and SAP-$1^{-/-}$ cultures, completely blocked the induction of Foxp3 expression in WT cultures (Figure 8.2 bottom row). These data are consistent with the DN Raf data suggesting that ERK signalling is required for the peripheral conversion process.

However there are several concerns regarding these data. In these experiments the WT data is significantly higher than in previous experiments that looked at the role of ERK signalling through the use of the MEK inhibitor UO126 (see section 6.9.1.2). Therefore while the induction of Foxp3 expression is lower in SAP-$1^{-/-}$ and DN Raf cells, the induced level is equivalent to that previously observed for WT cells and as such it is not possible to conclude that these cells are defective in their ability to undergo peripheral conversion. Furthermore the variable background expression of Foxp3 may indicate some degree of contamination of the naïve cells with regulatory T cells. Thus using additional markers such as CD45RB may help reduce the variability of basal Foxp3 expression.

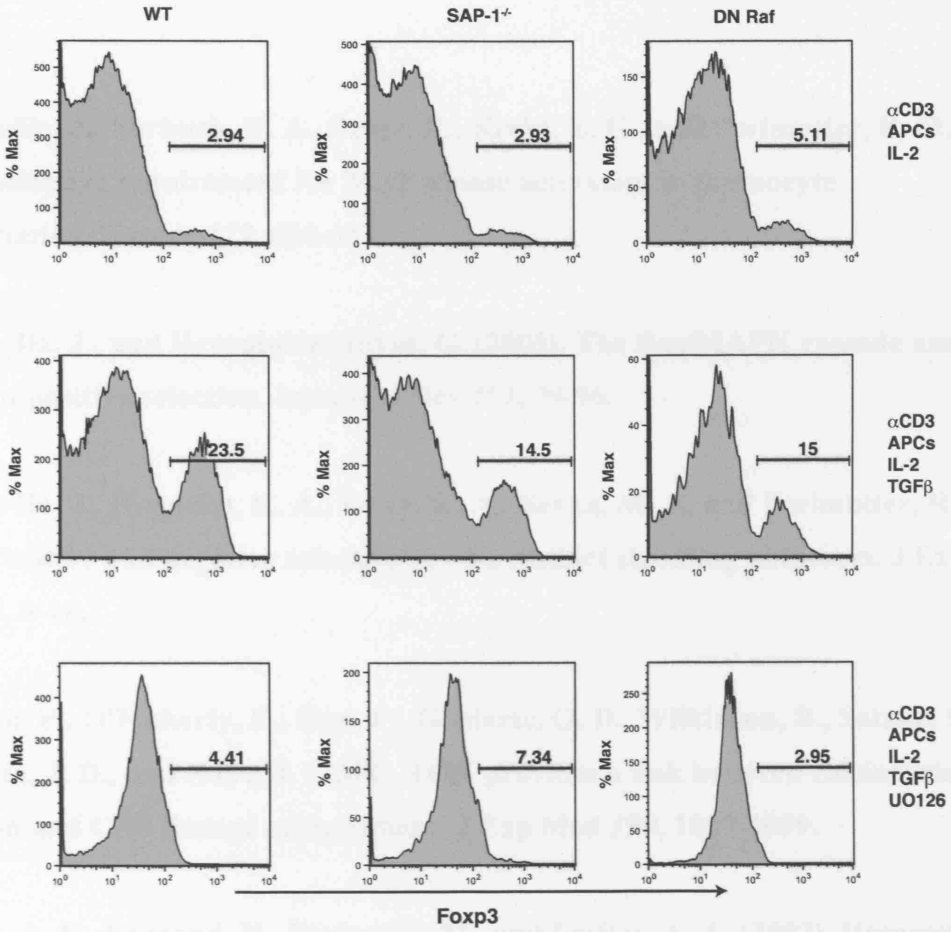


Figure 8.2. SAP-1 may be required for peripheral conversion. Cells were cultured over 3 days in either: αCD3,APCs and IL-2 (top row;, αCD3, APCs, IL-2 and TGFβ (middle row); or αCD3, APCs, IL-2, TGFβ and UO126 (bottom row). Foxp3 expression was assessed by intracellular staining.

References

Alberola-Ila, J., Forbush, K. A., Seger, R., Krebs, E. G., and Perlmutter, R. M. (1995). Selective requirement for MAP kinase activation in thymocyte differentiation. Nature *373*, 620-623.

Alberola-Ila, J., and Hernandez-Hoyos, G. (2003). The Ras/MAPK cascade and the control of positive selection. Immunol Rev *191*, 79-96.

Alberola-Ila, J., Hogquist, K. A., Swan, K. A., Bevan, M. J., and Perlmutter, R. M. (1996). Positive and negative selection invoke distinct signaling pathways. J Exp Med *184*, 9-18.

Aliahmad, P., O'Flaherty, E., Han, P., Goularte, O. D., Wilkinson, B., Satake, M., Molkentin, J. D., and Kaye, J. (2004). TOX provides a link between calcineurin activation and CD8 lineage commitment. J Exp Med *199*, 1089-1099.

Almeida, A. R., Legrand, N., Papiernik, M., and Freitas, A. A. (2002). Homeostasis of peripheral CD4+ T cells: IL-2R alpha and IL-2 shape a population of regulatory cells that controls CD4+ T cell numbers. J Immunol *169*, 4850-4860.

Apostolou, I., Sarukhan, A., Klein, L., and von Boehmer, H. (2002). Origin of regulatory T cells with known specificity for antigen. Nat Immunol *3*, 756-763.

Arsenian, S., Weinhold, B., Oelgeschlager, M., Ruther, U., and Nordheim, A. (1998). Serum response factor is essential for mesoderm formation during mouse embryogenesis. Embo J *17*, 6289-6299.

Asano, M., Toda, M., Sakaguchi, N., and Sakaguchi, S. (1996). Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med *184*, 387-396.

Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L., and Powrie, F. (1999). An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med *190*, 995-1004.

Ayadi, A., Zheng, H., Sobieszczuk, P., Buchwalter, G., Moerman, P., Alitalo, K., and Wasylyk, B. (2001). Net-targeted mutant mice develop a vascular phenotype and up-regulate egr-1. Embo J *20*, 5139-5152.

Bain, G., Cravatt, C. B., Loomans, C., Alberola-Ila, J., Hedrick, S. M., and Murre, C. (2001). Regulation of the helix-loop-helix proteins, E2A and Id3, by the Ras-ERK MAPK cascade. Nat Immunol *2*, 165-171.

Bain, G., Engel, I., Robanus Maandag, E. C., te Riele, H. P., Voland, J. R., Sharp, L. L., Chun, J., Huey, B., Pinkel, D., and Murre, C. (1997). E2A deficiency leads to abnormalities in alphabeta T-cell development and to rapid development of T-cell lymphomas. Mol Cell Biol *17*, 4782-4791.

Bain, G., Quong, M. W., Soloff, R. S., Hedrick, S. M., and Murre, C. (1999). Thymocyte maturation is regulated by the activity of the helix-loop-helix protein, E47. J Exp Med *190*, 1605-1616.

Bancroft, J. and M. Gamble (2002). Theory and Practice of Histological Techniques, 5th edn: Churchill Livingstone, New York).

Banz, A., Peixoto, A., Pontoux, C., Cordier, C., Rocha, B., and Papiernik, M. (2003). A unique subpopulation of CD4+ regulatory T cells controls wasting disease, IL-10 secretion and T cell homeostasis. Eur J Immunol *33*, 2419-2428.

Baron, A., Hafen, K., and von Boehmer, H. (1994). A human CD4 transgene rescues CD4-CD8+ cells in beta 2-microglobulin-deficient mice. Eur J Immunol *24*, 1933-1936.

Basson, M. A., and Zamoyska, R. (2000). The CD4/CD8 lineage decision: integration of signalling pathways. Immunol Today *21*, 509-514.

Belaguli, N. S., Sepulveda, J. L., Nigam, V., Charron, F., Nemer, M., and Schwartz, R. J. (2000). Cardiac tissue enriched factors serum response factor and GATA-4 are mutual coregulators. Mol Cell Biol *20*, 7550-7558.

Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M., and Sacks, D. L. (2002). CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature *420*, 502-507.

Bensinger, S. J., Bandeira, A., Jordan, M. S., Caton, A. J., and Laufer, T. M. (2001). Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells. J Exp Med *194*, 427-438.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L., and Kuchroo, V. K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature *441*, 235-238.

Bettini, M., Xi, H., Milbrandt, J., and Kersh, G. J. (2002). Thymocyte development in early growth response gene 1-deficient mice. J Immunol *169*, 1713-1720.

Bevan, M. J. (1977). In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. Nature *269*, 417-418.

Bluestone, J. A. (1997). Is CTLA-4 a master switch for peripheral T cell tolerance? J Immunol *158*, 1989-1993.

Bodor, J., Fehervari, Z., Diamond, B., and Sakaguchi, S. (2007). ICER/CREM-mediated transcriptional attenuation of IL-2 and its role in suppression by regulatory T cells. Eur J Immunol *37*, 884-895.

Bogoyevitch, M. A., and Court, N. W. (2004). Counting on mitogen-activated protein kinases--ERKs 3, 4, 5, 6, 7 and 8. Cell Signal *16*, 1345-1354.

Bogoyevitch, M. A., Marshall, C. J., and Sugden, P. H. (1995). Hypertrophic agonists stimulate the activities of the protein kinases c-Raf and A-Raf in cultured ventricular myocytes. J Biol Chem *270*, 26303-26310.

Bommhardt, U., Basson, M. A., Krummrei, U., and Zamoyska, R. (1999). Activation of the extracellular signal-related kinase/mitogen-activated protein kinase pathway discriminates CD4 versus CD8 lineage commitment in the thymus. J Immunol *163*, 715-722.

Borgulya, P., Kishi, H., Muller, U., Kirberg, J., and von Boehmer, H. (1991). Development of the CD4 and CD8 lineage of T cells: instruction versus selection. Embo J *10*, 913-918.

Bosselut, R. (2004). CD4/CD8-lineage differentiation in the thymus: from nuclear effectors to membrane signals. Nat Rev Immunol *4*, 529-540.

Boyton, R. J., and Altmann, D. M. (2002). Is selection for TCR affinity a factor in cytokine polarization? Trends Immunol *23*, 526-529.

Brugnera, E., Bhandoola, A., Cibotti, R., Yu, Q., Guinter, T. I., Yamashita, Y., Sharrow, S. O., and Singer, A. (2000). Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. Immunity *13*, 59-71.

Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., Wilkinson, J. E., Galas, D., Ziegler, S. F., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet *27*, 68-73.

Buchwalter, G., Gross, C., and Wasylyk, B. (2004). Ets ternary complex transcription factors. Gene *324*, 1-14.

Buchwalter, G., Gross, C., and Wasylyk, B. (2005). The ternary complex factor Net regulates cell migration through inhibition of PAI-1 expression. Mol Cell Biol *25*, 10853-10862.

Cabarrocas, J., Cassan, C., Magnusson, F., Piaggio, E., Mars, L., Derbinski, J., Kyewski, B., Gross, D. A., Salomon, B. L., Khazaie, K., *et al.* (2006). Foxp3+ CD25+ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage. Proc Natl Acad Sci U S A *103*, 8453-8458.

Calnan, B. J., Szychowski, S., Chan, F. K., Cado, D., and Winoto, A. (1995). A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. Immunity *3*, 273-282.

Carter, J. D., Calabrese, G. M., Naganuma, M., and Lorenz, U. (2005). Deficiency of the Src homology region 2 domain-containing phosphatase 1 (SHP-1) causes enrichment of CD4+CD25+ regulatory T cells. J Immunol *174*, 6627-6638.

Carter, J. H., Lefebvre, J. M., Wiest, D. L., and Tourtellotte, W. G. (2007). Redundant role for early growth response transcriptional regulators in thymocyte differentiation and survival. J Immunol *178*, 6796-6805.

Cederbom, L., Hall, H., and Ivars, F. (2000). CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. Eur J Immunol *30*, 1538-1543.

Cesari, F., Brecht, S., Vintersten, K., Vuong, L. G., Hofmann, M., Klingel, K., Schnorr, J. J., Arsenian, S., Schild, H., Herdegen, T., *et al.* (2004). Mice deficient for the ets transcription factor elk-1 show normal immune responses and mildly impaired neuronal gene activation. Mol Cell Biol *24*, 294-305.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature *410*, 37-40.

Chen, C. Y., and Schwartz, R. J. (1996). Recruitment of the tinman homolog Nkx-2.5 by serum response factor activates cardiac alpha-actin gene transcription. Mol Cell Biol *16*, 6372-6384.

Chen, J., Fujii, K., Zhang, L., Roberts, T., and Fu, H. (2001). Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. Proc Natl Acad Sci U S A *98*, 7783-7788.

Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G., and Wahl, S. M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med *198*, 1875-1886.

Cheng, A. M., and Chan, A. C. (1997). Protein tyrosine kinases in thymocyte development. Curr Opin Immunol *9*, 528-533.

Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., 2nd, Cox, A. D., and Philips, M. R. (2002). Ras signalling on the endoplasmic reticulum and the Golgi. Nat Cell Biol *4*, 343-350.

Clements, J. L., John, S. A., and Garrett-Sinha, L. A. (2006). Impaired generation of CD8+ thymocytes in Ets-1-deficient mice. J Immunol *177*, 905-912.

Clements, J. L., Yang, B., Ross-Barta, S. E., Eliason, S. L., Hrstka, R. F., Williamson, R. A., and Koretzky, G. A. (1998). Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. Science *281*, 416-419.

Cleverley, S., Henning, S., and Cantrell, D. (1999). Inhibition of Rho at different stages of thymocyte development gives different perspectives on Rho function. Curr Biol *9*, 657-660.

Collins, S., Wolfraim, L. A., Drake, C. G., Horton, M. R., and Powell, J. D. (2006). Cutting Edge: TCR-induced NAB2 enhances T cell function by coactivating IL-2 transcription. J Immunol *177*, 8301-8305.

Constant, S. L., Dong, C., Yang, D. D., Wysk, M., Davis, R. J., and Flavell, R. A. (2000). JNK1 is required for T cell-mediated immunity against Leishmania major infection. J Immunol *165*, 2671-2676.

Coombes, J. L., Siddiqui, K. R., Arancibia-Carcamo, C. V., Hall, J., Sun, C. M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med *204*, 1757-1764.

Costello, P. S., Nicolas, R. H., Watanabe, Y., Rosewell, I., and Treisman, R. (2004). Ternary complex factor SAP-1 is required for Erk-mediated thymocyte positive selection. Nat Immunol *5*, 289-298.

Countaway, J. L., Nairn, A. C., and Davis, R. J. (1992). Mechanism of desensitization of the epidermal growth factor receptor protein-tyrosine kinase. J Biol Chem *267*, 1129-1140.

Criqui-Filipe, P., Ducret, C., Maira, S. M., and Wasylyk, B. (1999). Net, a negative Ras-switchable TCF, contains a second inhibition domain, the CID, that mediates repression through interactions with CtBP and de-acetylation. Embo J *18*, 3392-3403.

Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., *et al.* (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature *421*, 744-748.

Dai, Z., Li, Q., Wang, Y., Gao, G., Diggs, L. S., Tellides, G., and Lakkis, F. G. (2004). CD4+CD25+ regulatory T cells suppress allograft rejection mediated by memory CD8+ T cells via a CD30-dependent mechanism. J Clin Invest *113*, 310-317.

Dalton, S., and Treisman, R. (1992). Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell *68*, 597-612.

Daniels, M. A., Teixeiro, E., Gill, J., Hausmann, B., Roubaty, D., Holmberg, K., Werlen, G., Hollander, G. A., Gascoigne, N. R., and Palmer, E. (2006). Thymic

selection threshold defined by compartmentalization of Ras/MAPK signalling. Nature *444*, 724-729.

Davidson, T. S., DiPaolo, R. J., Andersson, J., and Shevach, E. M. (2007). Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. J Immunol *178*, 4022-4026.

Davis, C. B., Killeen, N., Crooks, M. E., Raulet, D., and Littman, D. R. (1993). Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes. Cell *73*, 237-247.

Delgado, P., Fernandez, E., Dave, V., Kappes, D., and Alarcon, B. (2000). CD3delta couples T-cell receptor signalling to ERK activation and thymocyte positive selection. Nature *406*, 426-430.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995). Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science *267*, 682-685.

DeSilva, D. R., Jones, E. A., Favata, M. F., Jaffee, B. D., Magolda, R. L., Trzaskos, J. M., and Scherle, P. A. (1998). Inhibition of mitogen-activated protein kinase kinase blocks T cell proliferation but does not induce or prevent anergy. J Immunol *160*, 4175-4181.

Dong, C., Davis, R. J., and Flavell, R. A. (2002). MAP kinases in the immune response. Annu Rev Immunol *20*, 55-72.

Dong, C., Yang, D. D., Tournier, C., Whitmarsh, A. J., Xu, J., Davis, R. J., and Flavell, R. A. (2000). JNK is required for effector T-cell function but not for T-cell activation. Nature *405*, 91-94.

Dong, C., Yang, D. D., Wysk, M., Whitmarsh, A. J., Davis, R. J., and Flavell, R. A. (1998). Defective T cell differentiation in the absence of Jnk1. Science *282*, 2092-2095.

Dower, N. A., Stang, S. L., Bottorff, D. A., Ebinu, J. O., Dickie, P., Ostergaard, H. L., and Stone, J. C. (2000). RasGRP is essential for mouse thymocyte differentiation and TCR signaling. Nat Immunol *1*, 317-321.

Downward, J., Graves, J. D., Warne, P. H., Rayter, S., and Cantrell, D. A. (1990). Stimulation of p21ras upon T-cell activation. Nature *346*, 719-723.

Ducret, C., Maira, S. M., Lutz, Y., and Wasylyk, B. (2000). The ternary complex factor Net contains two distinct elements that mediate different responses to MAP kinase signalling cascades. Oncogene *19*, 5063-5072.

Dudley, E. C., Petrie, H. T., Shah, L. M., Owen, M. J., and Hayday, A. C. (1994). T cell receptor beta chain gene rearrangement and selection during thymocyte development in adult mice. Immunity *1*, 83-93.

Ebinu, J. O., Stang, S. L., Teixeira, C., Bottorff, D. A., Hooton, J., Blumberg, P. M., Barry, M., Bleakley, R. C., Ostergaard, H. L., and Stone, J. C. (2000). RasGRP links T-cell receptor signaling to Ras. Blood *95*, 3199-3203.

Ehlers, M., Laule-Kilian, K., Petter, M., Aldrian, C. J., Grueter, B., Wurch, A., Yoshida, N., Watanabe, T., Satake, M., and Steimle, V. (2003). Morpholino antisense oligonucleotide-mediated gene knockdown during thymocyte development reveals role for Runx3 transcription factor in CD4 silencing during development of CD4-/CD8+ thymocytes. J Immunol *171*, 3594-3604.

Elion, E. A. (2001). The Ste5p scaffold. J Cell Sci *114*, 3967-3978.

Engel, I., and Murre, C. (2001). The function of E- and Id proteins in lymphocyte development. Nat Rev Immunol *1*, 193-199.

Fahlen, L., Read, S., Gorelik, L., Hurst, S. D., Coffman, R. L., Flavell, R. A., and Powrie, F. (2005). T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. J Exp Med *201*, 737-746.

Fantini, M. C., Becker, C., Monteleone, G., Pallone, F., Galle, P. R., and Neurath, M. F. (2004). Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol *172*, 5149-5153.

Fehervari, Z., and Sakaguchi, S. (2004). Control of Foxp3+ CD25+CD4+ regulatory cell activation and function by dendritic cells. Int Immunol *16*, 1769-1780.

Fields, P. E., Gajewski, T. F., and Fitch, F. W. (1996). Blocked Ras activation in anergic CD4+ T cells. Science *271*, 1276-1278.

Fischer, A. M., Katayama, C. D., Pages, G., Pouyssegur, J., and Hedrick, S. M. (2005). The role of erk1 and erk2 in multiple stages of T cell development. Immunity *23*, 431-443.

Fleige, A., Alberti, S., Grobe, L., Frischmann, U., Geffers, R., Muller, W., Nordheim, A., and Schippers, A. (2007). Serum response factor contributes selectively to lymphocyte development. J Biol Chem *282*, 24320-24328.

Fontenot, J. D., Gavin, M. A., and Rudensky, A. Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol *4*, 330-336.

Fontenot, J. D., Rasmussen, J. P., Gavin, M. A., and Rudensky, A. Y. (2005a). A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat Immunol *6*, 1142-1151.

Fontenot, J. D., Rasmussen, J. P., Williams, L. M., Dooley, J. L., Farr, A. G., and Rudensky, A. Y. (2005b). Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity *22*, 329-341.

Fontenot, J. D., and Rudensky, A. Y. (2005). A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. Nat Immunol *6*, 331-337.

Ganiatsas, S., Kwee, L., Fujiwara, Y., Perkins, A., Ikeda, T., Labow, M. A., and Zon, L. I. (1998). SEK1 deficiency reveals mitogen-activated protein kinase cascade crossregulation and leads to abnormal hepatogenesis. Proc Natl Acad Sci U S A *95*, 6881-6886.

Gartner, F., Alt, F. W., Monroe, R., Chu, M., Sleckman, B. P., Davidson, L., and Swat, W. (1999). Immature thymocytes employ distinct signaling pathways for allelic exclusion versus differentiation and expansion. Immunity *10*, 537-546.

Gavin, M. A., Rasmussen, J. P., Fontenot, J. D., Vasta, V., Manganiello, V. C., Beavo, J. A., and Rudensky, A. Y. (2007). Foxp3-dependent programme of regulatory T-cell differentiation. Nature *445*, 771-775.

Genot, E., Cleverley, S., Henning, S., and Cantrell, D. (1996). Multiple p21ras effector pathways regulate nuclear factor of activated T cells. Embo J *15*, 3923-3933.

Germain, R. N. (2002). T-cell development and the CD4-CD8 lineage decision. Nat Rev Immunol *2*, 309-322.

Gille, H., Kortenjann, M., Thomae, O., Moomaw, C., Slaughter, C., Cobb, M. H., and Shaw, P. E. (1995). ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. Embo J *14*, 951-962.

Gineitis, D., and Treisman, R. (2001). Differential usage of signal transduction pathways defines two types of serum response factor target gene. J Biol Chem *276*, 24531-24539.

Giovane, A., Pintzas, A., Maira, S. M., Sobieszczuk, P., and Wasylyk, B. (1994). Net, a new ets transcription factor that is activated by Ras. Genes Dev *8*, 1502-1513.

Giroux, S., Tremblay, M., Bernard, D., Cardin-Girard, J. F., Aubry, S., Larouche, L., Rousseau, S., Huot, J., Landry, J., Jeannotte, L., and Charron, J. (1999).

Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. Curr Biol *9*, 369-372.

Glimcher, L. H., and Murphy, K. M. (2000). Lineage commitment in the immune system: the T helper lymphocyte grows up. Genes Dev *14*, 1693-1711.

Gondek, D. C., Lu, L. F., Quezada, S. A., Sakaguchi, S., and Noelle, R. J. (2005). Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. J Immunol *174*, 1783-1786.

Gong, Q., Cheng, A. M., Akk, A. M., Alberola-Ila, J., Gong, G., Pawson, T., and Chan, A. C. (2001). Disruption of T cell signaling networks and development by Grb2 haploid insufficiency. Nat Immunol *2*, 29-36.

Greaves, D. R., Wilson, F. D., Lang, G., and Kioussis, D. (1989). Human CD2 3'-flanking sequences confer high-level, T cell-specific, position-independent gene expression in transgenic mice. Cell *56*, 979-986.

Grossman, W. J., Verbsky, J. W., Barchet, W., Colonna, M., Atkinson, J. P., and Ley, T. J. (2004a). Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity *21*, 589-601.

Grossman, W. J., Verbsky, J. W., Tollefsen, B. L., Kemper, C., Atkinson, J. P., and Ley, T. J. (2004b). Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. Blood *104*, 2840-2848.

Groves, T., Smiley, P., Cooke, M. P., Forbush, K., Perlmutter, R. M., and Guidos, C. J. (1996). Fyn can partially substitute for Lck in T lymphocyte development. Immunity *5*, 417-428.

Gurney, A. L., Marsters, S. A., Huang, R. M., Pitti, R. M., Mark, D. T., Baldwin, D. T., Gray, A. M., Dowd, A. D., Brush, A. D., Heldens, A. D., *et al.* (1999). Identification of a new member of the tumor necrosis factor family and its receptor, a human ortholog of mouse GITR. Curr Biol *9*, 215-218.

Hashimoto, K., Sohn, S. J., Levin, S. D., Tada, T., Perlmutter, R. M., and Nakayama, T. (1996). Requirement for p56lck tyrosine kinase activation in T cell receptor-mediated thymic selection. J Exp Med *184*, 931-943.

Hatano, N., Mori, Y., Oh-hora, M., Kosugi, A., Fujikawa, T., Nakai, N., Niwa, H., Miyazaki, J., Hamaoka, T., and Ogata, M. (2003). Essential role for ERK2 mitogen-activated protein kinase in placental development. Genes Cells *8*, 847-856.

Hayday, A. C. (2000). [gamma][delta] cells: a right time and a right place for a conserved third way of protection. Annu Rev Immunol *18*, 975-1026.

Hayday, A. C., and Pennington, D. J. (2007). Key factors in the organized chaos of early T cell development. Nat Immunol *8*, 137-144.

He, X., He, X., Dave, V. P., Zhang, Y., Hua, X., Nicolas, E., Xu, W., Roe, B. A., and Kappes, D. J. (2005). The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. Nature *433*, 826-833.

He, X., and Kappes, D. J. (2006). CD4/CD8 lineage commitment: light at the end of the tunnel? Curr Opin Immunol *18*, 135-142.

Hickman, S. P., Yang, J., Thomas, R. M., Wells, A. D., and Turka, L. A. (2006). Defective activation of protein kinase C and Ras-ERK pathways limits IL-2 production and proliferation by CD4+CD25+ regulatory T cells. J Immunol *177*, 2186-2194.

Hollenhorst, P. C., Jones, D. A., and Graves, B. J. (2004). Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. Nucleic Acids Res *32*, 5693-5702.

Hollenhorst, P. C., Shah, A. A., Hopkins, C., and Graves, B. J. (2007). Genome-wide analyses reveal properties of redundant and specific promoter occupancy within the ETS gene family. Genes Dev *21*, 1882-1894.

Hori, S., Haury, M., Coutinho, A., and Demengeot, J. (2002). Specificity requirements for selection and effector functions of CD25+4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. Proc Natl Acad Sci U S A *99*, 8213-8218.

Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. Science *299*, 1057-1061.

Hsieh, C. S., Liang, Y., Tyznik, A. J., Self, S. G., Liggitt, D., and Rudensky, A. Y. (2004). Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. Immunity *21*, 267-277.

Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A., and Murphy, K. M. (1993). Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science *260*, 547-549.

Hsieh, C. S., Zheng, Y., Liang, Y., Fontenot, J. D., and Rudensky, A. Y. (2006). An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. Nat Immunol *7*, 401-410.

Huang, C. T., Workman, C. J., Flies, D., Pan, X., Marson, A. L., Zhou, G., Hipkiss, E. L., Ravi, S., Kowalski, J., Levitsky, H. I., *et al.* (2004). Role of LAG-3 in regulatory T cells. Immunity *21*, 503-513.

Hue, S., Ahern, P., Buonocore, S., Kullberg, M. C., Cua, D. J., McKenzie, B. S., Powrie, F., and Maloy, K. J. (2006). Interleukin-23 drives innate and T cell-mediated intestinal inflammation. J Exp Med *203*, 2473-2483.

Huehn, J., Siegmund, K., Lehmann, J. C., Siewert, C., Haubold, U., Feuerer, M., Debes, G. F., Lauber, J., Frey, O., Przybylski, G. K., *et al.* (2004). Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. J Exp Med *199*, 303-313.

Huseby, E. S., Crawford, F., White, J., Kappler, J., and Marrack, P. (2003). Negative selection imparts peptide specificity to the mature T cell repertoire. Proc Natl Acad Sci U S A *100*, 11565-11570.

Huser, M., Luckett, J., Chiloeches, A., Mercer, K., Iwobi, M., Giblett, S., Sun, X. M., Brown, J., Marais, R., and Pritchard, C. (2001). MEK kinase activity is not necessary for Raf-1 function. Embo J *20*, 1940-1951.

Iezzi, G., Scotet, E., Scheidegger, D., and Lanzavecchia, A. (1999). The interplay between the duration of TCR and cytokine signaling determines T cell polarization. Eur J Immunol *29*, 4092-4101.

Iritani, B. M., Alberola-Ila, J., Forbush, K. A., and Perimutter, R. M. (1999). Distinct signals mediate maturation and allelic exclusion in lymphocyte progenitors. Immunity *10*, 713-722.

Irving, B. A., Alt, F. W., and Killeen, N. (1998). Thymocyte development in the absence of pre-T cell receptor extracellular immunoglobulin domains. Science *280*, 905-908.

Itano, A., Kioussis, D., and Robey, E. (1994). Stochastic component to development of class I major histocompatibility complex-specific T cells. Proc Natl Acad Sci U S A *91*, 220-224.

Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F., and Sakaguchi, S. (1999). Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J Immunol *162*, 5317-5326.

Jaffe, A. B., Aspenstrom, P., and Hall, A. (2004). Human CNK1 acts as a scaffold protein, linking Rho and Ras signal transduction pathways. Mol Cell Biol *24*, 1736-1746.

Janknecht, R., Ernst, W. H., Pingoud, V., and Nordheim, A. (1993). Activation of ternary complex factor Elk-1 by MAP kinases. Embo J *12*, 5097-5104.

Janknecht, R., Zinck, R., Ernst, W. H., and Nordheim, A. (1994). Functional dissection of the transcription factor Elk-1. Oncogene *9*, 1273-1278.

Jenkins, M. K., Pardoll, D. M., Mizuguchi, J., Quill, H., and Schwartz, R. H. (1987). T-cell unresponsiveness in vivo and in vitro: fine specificity of induction and molecular characterization of the unresponsive state. Immunol Rev *95*, 113-135.

Jiang, Q., Su, H., Knudsen, G., Helms, W., and Su, L. (2006). Delayed functional maturation of natural regulatory T cells in the medulla of postnatal thymus: role of TSLP. BMC immunol 7:6 1-14.

Johnson, G. L., and Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science *298*, 1911-1912.

Jordan, M. S., Boesteanu, A., Reed, A. J., Petrone, A. L., Holenbeck, A. E., Lerman, M. A., Naji, A., and Caton, A. J. (2001). Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol *2*, 301-306.

Jorritsma, P. J., Brogdon, J. L., and Bottomly, K. (2003). Role of TCR-induced extracellular signal-regulated kinase activation in the regulation of early IL-4 expression in naive CD4+ T cells. J Immunol *170*, 2427-2434.

Kamakura, S., Moriguchi, T., and Nishida, E. (1999). Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. J Biol Chem *274*, 26563-26571.

Kang, J., Volkmann, A., and Raulet, D. H. (2001). Evidence that gammadelta versus alphabeta T cell fate determination is initiated independently of T cell receptor signaling. J Exp Med *193*, 689-698.

Kappes, D. J., He, X., and He, X. (2005). CD4-CD8 lineage commitment: an inside view. Nat Immunol *6*, 761-766.

Kawahata, K., Misaki, Y., Yamauchi, M., Tsunekawa, S., Setoguchi, K., Miyazaki, J., and Yamamoto, K. (2002). Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. J Immunol *168*, 4399-4405.

Khattri, R., Cox, T., Yasayko, S. A., and Ramsdell, F. (2003). An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol *4*, 337-342.

Kolch, W. (2005). Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat Rev Mol Cell Biol *6*, 827-837.

Koonpaew, S., Shen, S., Flowers, L., and Zhang, W. (2006). LAT-mediated signaling in CD4+CD25+ regulatory T cell development. J Exp Med *203*, 119-129.

Kortum, R. L., and Lewis, R. E. (2004). The molecular scaffold KSR1 regulates the proliferative and oncogenic potential of cells. Mol Cell Biol *24*, 4407-4416.

Kramer, S., Schimpl, A., and Hunig, T. (1995). Immunopathology of interleukin (IL) 2-deficient mice: thymus dependence and suppression by thymus-dependent cells with an intact IL-2 gene. J Exp Med *182*, 1769-1776.

Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M. C., and von Boehmer, H. (2005). Inducing and expanding regulatory T cell populations by foreign antigen. Nat Immunol *6*, 1219-1227.

Kulkarni, A. B., Huh, C. G., Becker, D., Geiser, A., Lyght, M., Flanders, K. C., Roberts, A. B., Sporn, M. B., Ward, J. M., and Karlsson, S. (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A *90*, 770-774.

Kumar, V., Bhardwaj, V., Soares, L., Alexander, J., Sette, A., and Sercarz, E. (1995). Major histocompatibility complex binding affinity of an antigenic

determinant is crucial for the differential secretion of interleukin 4/5 or interferon γ by T cells. Proc Natl Acad Sci USA 92, 9510-9514

Kuwahara, K., Barrientos, T., Pipes, G. C., Li, S., and Olson, E. N. (2005). Muscle-specific signaling mechanism that links actin dynamics to serum response factor. Mol Cell Biol *25*, 3173-3181.

Kwon, B., Yu, K. Y., Ni, J., Yu, G. L., Jang, I. K., Kim, Y. J., Xing, L., Liu, D., Wang, S. X., and Kwon, B. S. (1999). Identification of a novel activation-inducible protein of the tumor necrosis factor receptor superfamily and its ligand. J Biol Chem *274*, 6056-6061.

Ladi, E., Yin, X., Chtanova, T., and Robey, E. A. (2006). Thymic microenvironments for T cell differentiation and selection. Nat Immunol *7*, 338-343.

Lang, R., Hammer, M., and Mages, J. (2006). DUSP meet immunology: dual specificity MAPK phosphatases in control of the inflammatory response. J Immunol *177*, 7497-7504.

Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A., and Cua, D. J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med *201*, 233-240.

Layer, K., Lin, G., Nencioni, A., Hu, W., Schmucker, A., Antov, A. N., Li, X., Takamatsu, S., Chevassut, T., Dower, N. A., *et al.* (2003). Autoimmunity as the consequence of a spontaneous mutation in Rasgrp1. Immunity *19*, 243-255.

Lee, S. L., Wesselschmidt, R. L., Linette, G. P., Kanagawa, O., Russell, J. H., and Milbrandt, J. (1995). Unimpaired thymic and peripheral T cell death in mice lacking the nuclear receptor NGFI-B (Nur77). Science *269*, 532-535.

Lehmann, J., Huehn, J., de la Rosa, M., Maszyna, F., Kretschmer, U., Krenn, V., Brunner, M., Scheffold, A., and Hamann, A. (2002). Expression of the integrin

alpha Ebeta 7 identifies unique subsets of CD25+ as well as CD25- regulatory T cells. Proc Natl Acad Sci U S A *99*, 13031-13036.

Lerman, M. A., Larkin, J., 3rd, Cozzo, C., Jordan, M. S., and Caton, A. J. (2004). CD4+ CD25+ regulatory T cell repertoire formation in response to varying expression of a neo-self-antigen. J Immunol *173*, 236-244.

Letterio, J. J., and Roberts, A. B. (1998). Regulation of immune responses by TGF-beta. Annu Rev Immunol *16*, 137-161.

Li, M. O., Sanjabi, S., and Flavell, R. A. (2006). Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. Immunity *25*, 455-471.

Li, W., Whaley, C. D., Mondino, A., and Mueller, D. L. (1996). Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells. Science *271*, 1272-1276.

Lin, W., Haribhai, D., Relland, L. M., Truong, N., Carlson, M. R., Williams, C. B., and Chatila, T. A. (2007). Regulatory T cell development in the absence of functional Foxp3. Nat Immunol *8*, 359-368.

Liston, A., and Rudensky, A. Y. (2007). Thymic development and peripheral homeostasis of regulatory T cells. Curr Opin Immunol *19*, 176-185.

Liu, Y., Shepherd, E. G., and Nelin, L. D. (2007). MAPK phosphatases--regulating the immune response. Nat Rev Immunol *7*, 202-212.

Lohr, J., Knoechel, B., and Abbas, A. K. (2006). Regulatory T cells in the periphery. Immunol Rev *212*, 149-162.

Maggi, E., Parronchi, P., Manetti, R., Simonelli, C., Piccinni, M. P., Rugiu, F. S., De Carli, M., Ricci, M., and Romagnani, S. (1992). Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones. J Immunol *148*, 2142-2147.

Maillard, I., Fang, T., and Pear, W. S. (2005). Regulation of lymphoid development, differentiation, and function by the Notch pathway. Annu Rev Immunol *23*, 945-974.

Maira, S. M., Wurtz, J. M., and Wasylyk, B. (1996). Net (ERP/SAP2) one of the Ras-inducible TCFs, has a novel inhibitory domain with resemblance to the helix-loop-helix motif. Embo J *15*, 5849-5865.

Maloy, K. J., and Powrie, F. (2001). Regulatory T cells in the control of immune pathology. Nat Immunol *2*, 816-822.

Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M. P., Maggi, E., Trinchieri, G., and Romagnani, S. (1993). Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J Exp Med *177*, 1199-1204.

Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R., and Weaver, C. T. (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. Nature *441*, 231-234.

Marais, R., Light, Y., Paterson, H. F., Mason, C. S., and Marshall, C. J. (1997). Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. J Biol Chem *272*, 4378-4383.

Marais, R., Wynne, J., and Treisman, R. (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell *73*, 381-393.

Marie, J. C., Letterio, J. J., Gavin, M., and Rudensky, A. Y. (2005). TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. J Exp Med *201*, 1061-1067.

Marie, J. C., Liggitt, D., and Rudensky, A. Y. (2006). Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. Immunity *25*, 441-454.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell *80*, 179-185.

Maurice, D., Hooper, J., Lang, G., and Weston, K. (2007). c-Myb regulates lineage choice in developing thymocytes via its target gene Gata3. Embo J *26*, 3629-3640.

McCarty, N., Paust, S., Ikizawa, K., Dan, I., Li, X., and Cantor, H. (2005). Signaling by the kinase MINK is essential in the negative selection of autoreactive thymocytes. Nat Immunol *6*, 65-72.

McHugh, R. S., Whitters, M. J., Piccirillo, C. A., Young, D. A., Shevach, E. M., Collins, M., and Byrne, M. C. (2002). CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. Immunity *16*, 311-323.

Michie, A. M., and Zuniga-Pflucker, J. C. (2002). Regulation of thymocyte differentiation: pre-TCR signals and beta-selection. Semin Immunol *14*, 311-323.

Mikula, M., Schreiber, M., Husak, Z., Kucerova, L., Ruth, J., Wieser, R., Zatloukal, K., Beug, H., Wagner, E. F., and Baccarini, M. (2001). Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. Embo J *20*, 1952-1962.

Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, G. L., and Karin, M. (1994). Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science *266*, 1719-1723.

Miosge, L., and Zamoyska, R. (2007). Signalling in T-cell development: is it all location, location, location? Curr Opin Immunol *19*, 194-199.

Miralles, F., Posern, G., Zaromytidou, A. I., and Treisman, R. (2003). Actin dynamics control SRF activity by regulation of its coactivator MAL. Cell *113*, 329-342.

Miyazaki, T. (1997). Two distinct steps during thymocyte maturation from CD4-CD8- to CD4+CD8+ distinguished in the early growth response (Egr)-1 transgenic mice with a recombinase-activating gene-deficient background. J Exp Med *186*, 877-885.

Modigliani, Y., Bandeira, A., and Coutinho, A. (1996). A model for developmentally acquired thymus-dependent tolerance to central and peripheral antigens. Immunol Rev *149*, 155-120.

Mody, N., Leitch, J., Armstrong, C., Dixon, J., and Cohen, P. (2001). Effects of MAP kinase cascade inhibitors on the MKK5/ERK5 pathway. FEBS Lett *502*, 21-24.

Molina, T. J., Kishihara, K., Siderovski, D. P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C. J., Hartmann, K. U., Veillette, A., and et al. (1992). Profound block in thymocyte development in mice lacking p56lck. Nature *357*, 161-164.

Mombaerts, P., Anderson, S. J., Perlmutter, R. M., Mak, T. W., and Tonegawa, S. (1994). An activated lck transgene promotes thymocyte development in RAG-1 mutant mice. Immunity *1*, 261-267.

Mombaerts, P., Arnoldi, J., Russ, F., Tonegawa, S., and Kaufmann, S. H. (1993). Different roles of alpha beta and gamma delta T cells in immunity against an intracellular bacterial pathogen. Nature *365*, 53-56.

Mor, A., and Philips, M. R. (2006). Compartmentalized Ras/MAPK signaling. Annu Rev Immunol *24*, 771-800.

Morrison, D. K. (2001). KSR: a MAPK scaffold of the Ras pathway? J Cell Sci *114*, 1609-1612.

Mottet, C., Uhlig, H. H., and Powrie, F. (2003). Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol *170*, 3939-3943.

Mukasa, A., Hiramine, C., and Hojo, K. (1994). Generation and characterization of a continuous line of CD8+ suppressively regulatory T lymphocytes which down-regulates experimental autoimmune orchitis (EAO) in mice. Clin Exp Immunol *96*, 138-145.

Murai, K., and Treisman, R. (2002). Interaction of serum response factor (SRF) with the Elk-1 B box inhibits RhoA-actin signaling to SRF and potentiates transcriptional activation by Elk-1. Mol Cell Biol *22*, 7083-7092.

Murphy, C. A., Langrish, C. L., Chen, Y., Blumenschein, W., McClanahan, T., Kastelein, R. A., Sedgwick, J. D., and Cua, D. J. (2003). Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med *198*, 1951-1957.

Murphy, L. O., and Blenis, J. (2006). MAPK signal specificity: the right place at the right time. Trends Biochem Sci *31*, 268-275.

Murray, J. S. (1998) How the MHC selects Th1/Th2 immunity. Immunol Today 19, 157-162

Nakamura, K., Kitani, A., and Strober, W. (2001). Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J Exp Med *194*, 629-644.

Nawijn, M. C., Ferreira, R., Dingjan, G. M., Kahre, O., Drabek, D., Karis, A., Grosveld, F., and Hendriks, R. W. (2001). Enforced expression of GATA-3 during T cell development inhibits maturation of CD8 single-positive cells and induces thymic lymphoma in transgenic mice. J Immunol *167*, 715-723.

Negishi, I., Motoyama, N., Nakayama, K., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A. C., and Loh, D. Y. (1995). Essential role for ZAP-70 in both positive and negative selection of thymocytes. Nature *376*, 435-438.

Neilson, J. R., Winslow, M. M., Hur, E. M., and Crabtree, G. R. (2004). Calcineurin B1 is essential for positive but not negative selection during thymocyte development. Immunity *20*, 255-266.

Norton, J. D., Deed, R. W., Craggs, G., and Sablitzky, F. (1998). Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol *8*, 58-65.

O'Neill, E., Rushworth, L., Baccarini, M., and Kolch, W. (2004). Role of the kinase MST2 in suppression of apoptosis by the proto-oncogene product Raf-1. Science *306*, 2267-2270.

O'Shea, C. C., Crompton, T., Rosewell, I. R., Hayday, A. C., and Owen, M. J. (1996). Raf regulates positive selection. Eur J Immunol *26*, 2350-2355.

Pacholczyk, R., Ignatowicz, H., Kraj, P., and Ignatowicz, L. (2006). Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells. Immunity *25*, 249-259.

Pacholczyk, R., Kraj, P., and Ignatowicz, L. (2002). Peptide specificity of thymic selection of CD4+CD25+ T cells. J Immunol *168*, 613-620.

Pages, G., Guerin, S., Grall, D., Bonino, F., Smith, A., Anjuere, F., Auberger, P., and Pouyssegur, J. (1999). Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. Science *286*, 1374-1377.

Pai, S. Y., Truitt, M. L., Ting, C. N., Leiden, J. M., Glimcher, L. H., and Ho, I. C. (2003). Critical roles for transcription factor GATA-3 in thymocyte development. Immunity *19*, 863-875.

Palmer, E. (2003). Negative selection--clearing out the bad apples from the T-cell repertoire. Nat Rev Immunol *3*, 383-391.

Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., and Dong, C. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol *6*, 1133-1141.

Parlakian, A., Tuil, D., Hamard, G., Tavernier, G., Hentzen, D., Concordet, J. P., Paulin, D., Li, Z., and Daegelen, D. (2004). Targeted inactivation of serum response factor in the developing heart results in myocardial defects and embryonic lethality. Mol Cell Biol *24*, 5281-5289.

Pasare, C., and Medzhitov, R. (2003). Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science *299*, 1033-1036.

Peng, S. L., Madaio, M. P., Hayday, A. C., and Craft, J. (1996). Propagation and regulation of systemic autoimmunity by gammadelta T cells. J Immunol *157*, 5689-5698.

Pennington, D. J., Silva-Santos, B., Shires, J., Theodoridis, E., Pollitt, C., Wise, E. L., Tigelaar, R. E., Owen, M. J., and Hayday, A. C. (2003). The inter-relatedness and interdependence of mouse T cell receptor gammadelta+ and alphabeta+ cells. Nat Immunol *4*, 991-998.

Pennington, D. J., Silva-Santos, B., Silberzahn, T., Escorcio-Correia, M., Woodward, M. J., Roberts, S. J., Smith, A. L., Dyson, P. J., and Hayday, A. C. (2006). Early events in the thymus affect the balance of effector and regulatory T cells. Nature *444*, 1073-1077.

Petrie, H. T., and Zuniga-Pflucker, J. C. (2007). Zoned out: functional mapping of stromal signaling microenvironments in the thymus. Annu Rev Immunol *25*, 649-679.

Pfeiffer, C., Stein, J., Southwood, S., Ketelaar, H., Sette, A., and Bottomly, K. (1995). Altered Peptide Ligands Can Control CD4 T lymphocyte Differentiation In Vivo. J Ex Med 181, 1569-1574

Philippar, U., Schratt, G., Dieterich, C., Muller, J. M., Galgoczy, P., Engel, F. B., Keating, M. T., Gertler, F., Schule, R., Vingron, M., and Nordheim, A. (2004). The SRF target gene Fhl2 antagonizes RhoA/MAL-dependent activation of SRF. Mol Cell *16*, 867-880.

Piccirillo, C. A., Letterio, J. J., Thornton, A. M., McHugh, R. S., Mamura, M., Mizuhara, H., and Shevach, E. M. (2002). CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. J Exp Med *196*, 237-246.

Pipes, G. C., Creemers, E. E., and Olson, E. N. (2006). The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. Genes Dev *20*, 1545-1556.

Pivniouk, V., Tsitsikov, E., Swinton, P., Rathbun, G., Alt, F. W., and Geha, R. S. (1998). Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76. Cell *94*, 229-238.

Posern, G., and Treisman, R. (2006). Actin' together: serum response factor, its cofactors and the link to signal transduction. Trends Cell Biol *16*, 588-596.

Powrie, F., Carlino, J., Leach, M. W., Mauze, S., and Coffman, R. L. (1996). A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. J Exp Med *183*, 2669-2674.

Powrie, F., Correa-Oliveira, R., Mauze, S., and Coffman, R. L. (1994). Regulatory interactions between CD45RBhigh and CD45RBlow CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. J Exp Med *179*, 589-600.

Powrie, F., Leach, M. W., Mauze, S., Caddle, L. B., and Coffman, R. L. (1993). Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. Int Immunol *5*, 1461-1471.

Price, M. A., Hill, C., and Treisman, R. (1996). Integration of growth factor signals at the c-fos serum response element. Philos Trans R Soc Lond B Biol Sci *351*, 551-559.

Price, M. A., Rogers, A. E., and Treisman, R. (1995). Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET). Embo J *14*, 2589-2601.

Prinz, I., Sansoni, A., Kissenpfennig, A., Ardouin, L., Malissen, M., and Malissen, B. (2006). Visualization of the earliest steps of gammadelta T cell development in the adult thymus. Nat Immunol *7*, 995-1003.

Pritchard, C. A., Bolin, L., Slattery, R., Murray, R., and McMahon, M. (1996). Post-natal lethality and neurological and gastrointestinal defects in mice with targeted disruption of the A-Raf protein kinase gene. Curr Biol *6*, 614-617.

Rabizadeh, S., Xavier, R. J., Ishiguro, K., Bernabeortiz, J., Lopez-Ilasaca, M., Khokhlatchev, A., Mollahan, P., Pfeifer, G. P., Avruch, J., and Seed, B. (2004). The scaffold protein CNK1 interacts with the tumor suppressor RASSF1A and augments RASSF1A-induced cell death. J Biol Chem *279*, 29247-29254.

Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996). MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol Cell Biol *16*, 1247-1255.

Raman, M., Chen, W., and Cobb, M. H. (2007). Differential regulation and properties of MAPKs. Oncogene *26*, 3100-3112.

Read, S., Malmstrom, V., and Powrie, F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med *192*, 295-302.

Rincon, M., Enslen, H., Raingeaud, J., Recht, M., Zapton, T., Su, M. S., Penix, L. A., Davis, R. J., and Flavell, R. A. (1998a). Interferon-gamma expression by Th1

effector T cells mediated by the p38 MAP kinase signaling pathway. Embo J *17*, 2817-2829.

Rincon, M., Whitmarsh, A., Yang, D. D., Weiss, L., Derijard, B., Jayaraj, P., Davis, R. J., and Flavell, R. A. (1998b). The JNK pathway regulates the In vivo deletion of immature CD4(+)CD8(+) thymocytes. J Exp Med *188*, 1817-1830.

Rivera, R. R., Johns, C. P., Quan, J., Johnson, R. S., and Murre, C. (2000). Thymocyte selection is regulated by the helix-loop-helix inhibitor protein, Id3. Immunity *12*, 17-26.

Robey, E. A., Fowlkes, B. J., Gordon, J. W., Kioussis, D., von Boehmer, H., Ramsdell, F., and Axel, R. (1991). Thymic selection in CD8 transgenic mice supports an instructive model for commitment to a CD4 or CD8 lineage. Cell *64*, 99-107.

Ronchetti, S., Zollo, O., Bruscoli, S., Agostini, M., Bianchini, R., Nocentini, G., Ayroldi, E., and Riccardi, C. (2004). GITR, a member of the TNF receptor superfamily, is costimulatory to mouse T lymphocyte subpopulations. Eur J Immunol *34*, 613-622.

Rosat, J. P., MacDonald, H. R., and Louis, J. A. (1993). A role for gamma delta + T cells during experimental infection of mice with Leishmania major. J Immunol *150*, 550-555.

Saint-Ruf, C., Panigada, M., Azogui, O., Debey, P., von Boehmer, H., and Grassi, F. (2000). Different initiation of pre-TCR and gammadeltaTCR signalling. Nature *406*, 524-527.

Sakaguchi, S. (2000). Regulatory T cells: key controllers of immunologic self-tolerance. Cell *101*, 455-458.

Sakaguchi, S. (2004). Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol *22*, 531-562.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol *155*, 1151-1164.

Sakaguchi, S., Sakaguchi, N., Yoshitomi, H., Hata, H., Takahashi, T., and Nomura, T. (2006). Spontaneous development of autoimmune arthritis due to genetic anomaly of T cell signal transduction: Part 1. Semin Immunol *18*, 199-206.

Salinas, S., Briancon-Marjollet, A., Bossis, G., Lopez, M. A., Piechaczyk, M., Jariel-Encontre, I., Debant, A., and Hipskind, R. A. (2004). SUMOylation regulates nucleo-cytoplasmic shuttling of Elk-1. J Cell Biol *165*, 767-773.

Salomon, B., Lenschow, D. J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., and Bluestone, J. A. (2000). B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity *12*, 431-440.

Sansom, D. M., and Walker, L. S. (2006). The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. Immunol Rev *212*, 131-148.

Sato, T., Ohno, S., Hayashi, T., Sato, C., Kohu, K., Satake, M., and Habu, S. (2005). Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. Immunity *22*, 317-328.

Schnell, F. J., Zoller, A. L., Patel, S. R., Williams, I. R., and Kersh, G. J. (2006). Early growth response gene 1 provides negative feedback to inhibit entry of progenitor cells into the thymus. J Immunol *176*, 4740-4747.

Schramm, C., Huber, S., Protschka, M., Czochra, P., Burg, J., Schmitt, E., Lohse, A. W., Galle, P. R., and Blessing, M. (2004). TGFbeta regulates the CD4+CD25+ T-cell pool and the expression of Foxp3 in vivo. Int Immunol *16*, 1241-1249.

Schratt, G., Philippar, U., Hockemeyer, D., Schwarz, H., Alberti, S., and Nordheim, A. (2004). SRF regulates Bcl-2 expression and promotes cell survival during murine embryonic development. Embo J *23*, 1834-1844.

Schratt, G., Weinhold, B., Lundberg, A. S., Schuck, S., Berger, J., Schwarz, H., Weinberg, R. A., Ruther, U., and Nordheim, A. (2001). Serum response factor is required for immediate-early gene activation yet is dispensable for proliferation of embryonic stem cells. Mol Cell Biol *21*, 2933-2943.

Seddon, B., and Mason, D. (1999). Regulatory T cells in the control of autoimmunity: the essential role of transforming growth factor beta and interleukin 4 in the prevention of autoimmune thyroiditis in rats by peripheral CD4(+)CD45RC- cells and CD4(+)CD8(-) thymocytes. J Exp Med *189*, 279-288.

Sharp, L. L., Schwarz, D. A., Bott, C. M., Marshall, C. J., and Hedrick, S. M. (1997). The influence of the MAPK pathway on T cell lineage commitment. Immunity *7*, 609-618.

Sharrocks, A. D. (2001). The ETS-domain transcription factor family. Nat Rev Mol Cell Biol *2*, 827-837.

Sharrocks, A. D. (2002). Complexities in ETS-domain transcription factor function and regulation: lessons from the TCF (ternary complex factor) subfamily. The Colworth Medal Lecture. Biochem Soc Trans *30*, 1-9.

Shaw, P. E., Schroter, H., and Nordheim, A. (1989). The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-fos promoter. Cell *56*, 563-572.

Shevach, E. M. (2000). Regulatory T cells in autoimmmunity*. Annu Rev Immunol *18*, 423-449.

Shevach, E. M. (2002). CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol *2*, 389-400.

Shevach, E. M. (2004). Fatal attraction: tumors beckon regulatory T cells. Nat Med *10*, 900-901.

Shimizu, J., Yamazaki, S., and Sakaguchi, S. (1999). Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J Immunol *163*, 5211-5218.

Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y., and Sakaguchi, S. (2002). Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol *3*, 135-142.

Shore, P., Whitmarsh, A. J., Bhaskaran, R., Davis, R. J., Waltho, J. P., and Sharrocks, A. D. (1996). Determinants of DNA-binding specificity of ETS-domain transcription factors. Mol Cell Biol *16*, 3338-3349.

Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., and et al. (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature *359*, 693-699.

Silva-Santos, B., Pennington, D. J., and Hayday, A. C. (2005). Lymphotoxin-mediated regulation of gammadelta cell differentiation by alphabeta T cell progenitors. Science *307*, 925-928.

Singer, A., and Bosselut, R. (2004). CD4/CD8 coreceptors in thymocyte development, selection, and lineage commitment: analysis of the CD4/CD8 lineage decision. Adv Immunol *83*, 91-131.

Singh, R. P., La Cava, A., Wong, M., Ebling, F., and Hahn, B. H. (2007). CD8+ T cell-mediated suppression of autoimmunity in a murine lupus model of peptide-induced immune tolerance depends on Foxp3 expression. J Immunol *178*, 7649-7657.

Skeen, M. J., and Ziegler, H. K. (1993). Induction of murine peritoneal gamma/delta T cells and their role in resistance to bacterial infection. J Exp Med *178*, 971-984.

Skeen, M. J., and Ziegler, H. K. (1995). Activation of gamma delta T cells for production of IFN-gamma is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. J Immunol *154*, 5832-5841.

Song, X., Krelin, Y., Dvorkin, T., Bjorkdahl, O., Segal, S., Dinarello, C. A., Voronov, E., and Apte, R. N. (2005). CD11b+/Gr-1+ immature myeloid cells mediate suppression of T cells in mice bearing tumors of IL-1beta-secreting cells. J Immunol *175*, 8200-8208.

Speiser, D. E., Lees, R. K., Hengartner, H., Zinkernagel, R. M., and MacDonald, H. R. (1989). Positive and negative selection of T cell receptor V beta domains controlled by distinct cell populations in the thymus. J Exp Med *170*, 2165-2170.

Starr, T. K., Jameson, S. C., and Hogquist, K. A. (2003). Positive and negative selection of T cells. Annu Rev Immunol *21*, 139-176.

Stephens, G. L., McHugh, R. S., Whitters, M. J., Young, D. A., Luxenberg, D., Carreno, B. M., Collins, M., and Shevach, E. M. (2004). Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4+CD25+ T cells. J Immunol *173*, 5008-5020.

Strahl, T., Gille, H., and Shaw, P. E. (1996). Selective response of ternary complex factor Sap1a to different mitogen-activated protein kinase subgroups. Proc Natl Acad Sci U S A *93*, 11563-11568.

Sugawara, T., Moriguchi, T., Nishida, E., and Takahama, Y. (1998). Differential roles of ERK and p38 MAP kinase pathways in positive and negative selection of T lymphocytes. Immunity *9*, 565-574.

Sugimoto, T., Stewart, S., and Guan, K. L. (1997). The calcium/calmodulin-dependent protein phosphatase calcineurin is the major Elk-1 phosphatase. J Biol Chem *272*, 29415-29418.

Sun, G., Liu, X., Mercado, P., Jenkinson, S. R., Kypriotou, M., Feigenbaum, L., Galera, P., and Bosselut, R. (2005). The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection. Nat Immunol *6*, 373-381.

Suzuki, H., Zhou, Y. W., Kato, M., Mak, T. W., and Nakashima, I. (1999). Normal regulatory alpha/beta T cells effectively eliminate abnormally activated T cells lacking the interleukin 2 receptor beta in vivo. J Exp Med *190*, 1561-1572.

Swan, K. A., Alberola-Ila, J., Gross, J. A., Appleby, M. W., Forbush, K. A., Thomas, J. F., and Perlmutter, R. M. (1995). Involvement of p21ras distinguishes positive and negative selection in thymocytes. Embo J *14*, 276-285.

Swat, W., Shinkai, Y., Cheng, H. L., Davidson, L., and Alt, F. W. (1996). Activated Ras signals differentiation and expansion of CD4+8+ thymocytes. Proc Natl Acad Sci U S A *93*, 4683-4687.

Tai, X., Cowan, M., Feigenbaum, L., and Singer, A. (2005). CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. Nat Immunol *6*, 152-162.

Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J., and Sakaguchi, S. (1998). Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol *10*, 1969-1980.

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W., and Sakaguchi, S. (2000). Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med *192*, 303-310.

Tang, Q., Boden, E. K., Henriksen, K. J., Bour-Jordan, H., Bi, M., and Bluestone, J. A. (2004). Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. Eur J Immunol *34*, 2996-3005.

Tang, Q., Henriksen, K. J., Boden, E. K., Tooley, A. J., Ye, J., Subudhi, S. K., Zheng, X. X., Strom, T. B., and Bluestone, J. A. (2003). Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. J Immunol *171*, 3348-3352.

Taniuchi, I., Osato, M., Egawa, T., Sunshine, M. J., Bae, S. C., Komori, T., Ito, Y., and Littman, D. R. (2002). Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. Cell *111*, 621-633.

Thompson, C. B., and Allison, J. P. (1997). The emerging role of CTLA-4 as an immune attenuator. Immunity *7*, 445-450.

Thornton, A. M., Piccirillo, C. A., and Shevach, E. M. (2004). Activation requirements for the induction of CD4+CD25+ T cell suppressor function. Eur J Immunol *34*, 366-376.

Thornton, A. M., and Shevach, E. M. (1998). CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med *188*, 287-296.

Tivol, E. A., Borriello, F., Schweitzer, A. N., Lynch, W. P., Bluestone, J. A., and Sharpe, A. H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity *3*, 541-547.

Tone, M., Tone, Y., Adams, E., Yates, S. F., Frewin, M. R., Cobbold, S. P., and Waldmann, H. (2003). Mouse glucocorticoid-induced tumor necrosis factor receptor ligand is costimulatory for T cells. Proc Natl Acad Sci U S A *100*, 15059-15064.

Tournier, C., Whitmarsh, A. J., Cavanagh, J., Barrett, T., and Davis, R. J. (1997). Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. Proc Natl Acad Sci U S A *94*, 7337-7342.

Traverse, S., Gomez, N., Paterson, H., Marshall, C., and Cohen, P. (1992). Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. Biochem J *288 (Pt 2)*, 351-355.

Traverse, S., Seedorf, K., Paterson, H., Marshall, C. J., Cohen, P., and Ullrich, A. (1994). EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. Curr Biol *4*, 694-701.

Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. Curr Opin Genet Dev *4*, 96-101.

Tsang, J. Y., Camara, N. O., Eren, E., Schneider, H., Rudd, C., Lombardi, G., and Lechler, R. (2006). Altered proximal T cell receptor (TCR) signaling in human CD4+CD25+ regulatory T cells. J Leukoc Biol *80*, 145-151.

van Oers, N. S., Lowin-Kropf, B., Finlay, D., Connolly, K., and Weiss, A. (1996). alpha beta T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. Immunity *5*, 429-436.

Van Parijs, L., and Abbas, A. K. (1998). Homeostasis and self-tolerance in the immune system: turning lymphocytes off. Science *280*, 243-248.

van Santen, H. M., Benoist, C., and Mathis, D. (2004). Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. J Exp Med *200*, 1221-1230.

Veillette, A., Zuniga-Pflucker, J. C., Bolen, J. B., and Kruisbeek, A. M. (1989). Engagement of CD4 and CD8 expressed on immature thymocytes induces activation of intracellular tyrosine phosphorylation pathways. J Exp Med *170*, 1671-1680.

Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., and Stockinger, B. (2006). TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity *24*, 179-189.

Veldhoen, M., and Stockinger, B. (2006). TGFbeta1, a "Jack of all trades": the link with pro-inflammatory IL-17-producing T cells. Trends Immunol *27*, 358-361.

Vincent, M. S., Roessner, K., Lynch, D., Wilson, D., Cooper, S. M., Tschopp, J., Sigal, L. H., and Budd, R. C. (1996). Apoptosis of Fashigh CD4+ synovial T cells by borrelia-reactive Fas-ligand(high) gamma delta T cells in Lyme arthritis. J Exp Med *184*, 2109-2117.

von Boehmer, H., and Kisielow, P. (2006). Negative selection of the T-cell repertoire: where and when does it occur? Immunol Rev 209, 284-289.

Walker, L. S., Chodos, A., Eggena, M., Dooms, H., and Abbas, A. K. (2003). Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. J Exp Med *198*, 249-258.

Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N. (2001). Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. Cell *105*, 851-862.

Wang, Z., Wang, D. Z., Hockemeyer, D., McAnally, J., Nordheim, A., and Olson, E. N. (2004). Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. Nature *428*, 185-189.

Wasylyk, C., Criqui-Filipe, P., and Wasylyk, B. (2005). Sumoylation of the net inhibitory domain (NID) is stimulated by PIAS1 and has a negative effect on the transcriptional activity of Net. Oncogene *24*, 820-828.

Waterhouse, P., Penninger, J. M., Timms, E., Wakeham, A., Shahinian, A., Lee, K. P., Thompson, C. B., Griesser, H., and Mak, T. W. (1995). Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science *270*, 985-988.

Weaver, C. T., Harrington, L. E., Mangan, P. R., Gavrieli, M., and Murphy, K. M. (2006). Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity *24*, 677-688.

Weber, C. K., Slupsky, J. R., Herrmann, C., Schuler, M., Rapp, U. R., and Block, C. (2000). Mitogenic signaling of Ras is regulated by differential interaction with Raf isozymes. Oncogene *19*, 169-176.

Weigle, W.O. (1980) Analysis of autoimmunity through experimental models of thyroiditis and allergic encephalomyelitis. Adv Immunol 30, 159-273

Wekerle, H., Bradl, M., Linington, C., Kaab, G., and Kojima, K. (1996). The shaping of the brain-specific T lymphocyte repertoire in the thymus. Immunol Rev *149*, 231-243.

Wellbrock, C., Karasarides, M., and Marais, R. (2004). The RAF proteins take centre stage. Nat Rev Mol Cell Biol *5*, 875-885.

Whitmarsh, A. J., Yang, S. H., Su, M. S., Sharrocks, A. D., and Davis, R. J. (1997). Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. Mol Cell Biol *17*, 2360-2371.

Wiest, D. L., Yuan, L., Jefferson, J., Benveniste, P., Tsokos, M., Klausner, R. D., Glimcher, L. H., Samelson, L. E., and Singer, A. (1993). Regulation of T cell receptor expression in immature CD4+CD8+ thymocytes by p56lck tyrosine kinase: basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. J Exp Med *178*, 1701-1712.

Williams, L. M., and Rudensky, A. Y. (2007). Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol *8*, 277-284.

Wohlfert, E. A., Gorelik, L., Mittler, R., Flavell, R. A., and Clark, R. B. (2006). Cutting edge: deficiency in the E3 ubiquitin ligase Cbl-b results in a

multifunctional defect in T cell TGF-beta sensitivity in vitro and in vivo. J Immunol *176*, 1316-1320.

Wojnowski, L., Zimmer, A. M., Beck, T. W., Hahn, H., Bernal, R., Rapp, U. R., and Zimmer, A. (1997). Endothelial apoptosis in Braf-deficient mice. Nat Genet *16*, 293-297.

Wong, J., Mathis, D., and Benoist, C. (2007). TCR-based lineage tracing: no evidence for conversion of conventional into regulatory T cells in response to a natural self-antigen in pancreatic islets. J Exp Med *204*, 2039-2045.

Yajima, K., Nakamura, A., Sugahara, A., and Takai, T. (2003). FcgammaRIIB deficiency with Fas mutation is sufficient for the development of systemic autoimmune disease. Eur J Immunol *33*, 1020-1029.

Yamashita, M., Kimura, M., Kubo, M., Shimizu, C., Tada, T., Perlmutter, R. M., and Nakayama, T. (1999). T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. Proc Natl Acad Sci U S A *96*, 1024-1029.

Yang, D. D., Conze, D., Whitmarsh, A. J., Barrett, T., Davis, R. J., Rincon, M., and Flavell, R. A. (1998). Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. Immunity *9*, 575-585.

Yang, R., Cai, Z., Zhang, Y., Yutzy, W. H. t., Roby, K. F., and Roden, R. B. (2006). CD80 in immune suppression by mouse ovarian carcinoma-associated Gr-1+CD11b+ myeloid cells. Cancer Res *66*, 6807-6815.

Yang, S. H., Bumpass, D. C., Perkins, N. D., and Sharrocks, A. D. (2002). The ETS domain transcription factor Elk-1 contains a novel class of repression domain. Mol Cell Biol *22*, 5036-5046.

Yang, S. H., Jaffray, E., Hay, R. T., and Sharrocks, A. D. (2003). Dynamic interplay of the SUMO and ERK pathways in regulating Elk-1 transcriptional activity. Mol Cell *12*, 63-74.

Yang, S. H., and Sharrocks, A. D. (2004). SUMO promotes HDAC-mediated transcriptional repression. Mol Cell *13*, 611-617.

Yang, S. H., Shore, P., Willingham, N., Lakey, J. H., and Sharrocks, A. D. (1999). The mechanism of phosphorylation-inducible activation of the ETS-domain transcription factor Elk-1. Embo J *18*, 5666-5674.

Yang, S. H., Vickers, E., Brehm, A., Kouzarides, T., and Sharrocks, A. D. (2001). Temporal recruitment of the mSin3A-histone deacetylase corepressor complex to the ETS domain transcription factor Elk-1. Mol Cell Biol *21*, 2802-2814.

Yao, Y., Li, W., Wu, J., Germann, U. A., Su, M. S., Kuida, K., and Boucher, D. M. (2003). Extracellular signal-regulated kinase 2 is necessary for mesoderm differentiation. Proc Natl Acad Sci U S A *100*, 12759-12764.

Yates, P. R., Atherton, G. T., Deed, R. W., Norton, J. D., and Sharrocks, A. D. (1999). Id helix-loop-helix proteins inhibit nucleoprotein complex formation by the TCF ETS-domain transcription factors. Embo J *18*, 968-976.

Yin, X., Chtanova, T., Ladi, E., and Robey, E. A. (2006). Thymocyte motility: mutants, movies and migration patterns. Curr Opin Immunol *18*, 191-197.

Yoshitomi, H., Sakaguchi, N., Kobayashi, K., Brown, G. D., Tagami, T., Sakihama, T., Hirota, K., Tanaka, S., Nomura, T., Miki, I., *et al.* (2005). A role for fungal {beta}-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. J Exp Med *201*, 949-960.

Zaromytidou, A. I., Miralles, F., and Treisman, R. (2006). MAL and ternary complex factor use different mechanisms to contact a common surface on the serum response factor DNA-binding domain. Mol Cell Biol *26*, 4134-4148.

Zhang, S., and Kaplan, M. H. (2000). The p38 mitogen-activated protein kinase is required for IL-12-induced IFN-gamma expression. J Immunol *165*, 1374-1380.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998). LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell *92*, 83-92.

Zhang, W., Sommers, C. L., Burshtyn, D. N., Stebbins, C. C., DeJarnette, J. B., Trible, R. P., Grinberg, A., Tsay, H. C., Jacobs, H. M., Kessler, C. M., *et al.* (1999). Essential role of LAT in T cell development. Immunity *10*, 323-332.

Zhang, Y. L., and Dong, C. (2005). MAP kinases in immune responses. Cell Mol Immunol *2*, 20-27.

Zheng, H., Wasylyk, C., Ayadi, A., Abecassis, J., Schalken, J. A., Rogatsch, H., Wernert, N., Maira, S. M., Multon, M. C., and Wasylyk, B. (2003). The transcription factor Net regulates the angiogenic switch. Genes Dev *17*, 2283-2297.

Zheng, S. G., Wang, J., Wang, P., Gray, J. D., and Horwitz, D. A. (2007). IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. J Immunol *178*, 2018-2027.

Zheng, Y., and Rudensky, A. Y. (2007). Foxp3 in control of the regulatory T cell lineage. Nat Immunol *8*, 457-462.

Zhou, J., Chehab, R., Tkalcevic, J., Naylor, M. J., Harris, J., Wilson, T. J., Tsao, S., Tellis, I., Zavarsek, S., Xu, D., *et al.* (2005). Elf5 is essential for early embryogenesis and mammary gland development during pregnancy and lactation. Embo J *24*, 635-644.

Zhou, T., Cheng, J., Yang, P., Wang, Z., Liu, C., Su, X., Bluethmann, H., and Mountz, J. D. (1996). Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. J Exp Med *183*, 1879-1892.

Ziogas, A., Moelling, K., and Radziwill, G. (2005). CNK1 is a scaffold protein that regulates Src-mediated Raf-1 activation. J Biol Chem *280*, 24205-24211.

Zuniga-Pflucker, J. C., Schwartz, H. L., and Lenardo, M. J. (1993). Gene transcription in differentiating immature T cell receptor(neg) thymocytes resembles antigen-activated mature T cells. J Exp Med *178*, 1139-1149.